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Crosstalk Between MDM2 and Akt Signaling Pathway in Oncogenesis.

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CROSSTALK BETWEEN MDM2 AND AKT SIGNALING PATHWAY IN
ONCOGENESIS

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

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Dedication.

This work is dedicated to my family. Father, Mother and my sister – thank you for your support. I specially want to dedicate this work to my wife, Nithya, who was a constant source of motivation and support during my thesis writing.

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This work was supported by grants from Jeffress Memorial Trust and NCI to Dr Swati Deb (CA74172) and NIH to Sumitra Deb (CA70712) and Philip Morris International (04-I176-01). Special thanks must be given to my advisor Dr Swati Deb, committee members and other scientists at VCU for providing technical guidance. I would also like to thank Dr Gail Christie, Dr Shirley Taylor and the entire MBG program for all their support. Last but not least, thanks to my friends at the Massey Cancer Center, who made work easy with their wit and wisdom.

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List of Abbreviations:

4EBP1	4E Binding Protein 1
ARF	Alternate Reading Frame
BAD	BCL _{XL} /BCL2 associated death promoter homolog
Bcl2	B-cell lymphoma 2
CDK	Cyclin Dependent Kinase
CREB	cAMP Responsive Element Binding Protein 1
CTMP	Carboxy Terminal Modulating Protein
DMSO	Dimethylsulfoxide

DNA-PK	DNA dependent Protein Kinase
EF1 α	Elongation Factor 1 α
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular signal-regulated kinase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GPCR	G-Protein Coupled Receptor
GRK2	G-Protein Coupled Receptor Kinase
GSK3	Glycogen Synthase Kinase
HBSS	Hanks Balanced Salt Solution
IGF-1	Insulin-like Growth Factor
IGF-1R	Insulin-like Growth Factor-1 Receptor
IGFBP	Insulin-like growth factor binding protein
IL-8	Interleukin 8
ILK	Integrin Linked Kinase
IRS1	Insulin Receptor Substrate1
MDM2	Murine Double Minute 2.
MDR1	Multiple Drug Resistance 1
mTOR	Mamalian Target Of Rapamycin
NBS1	Nijmegen breakage syndrome1
NF- κ B	Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells.
NF- κ B2	Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells2
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDK1	Protein serine/threonine kinase 3'Phosphoinositide
PHLPP	PH domain leucine-rich repeat protein phosphatase
PI3K	Phosphoinositide 3 Kinase

PKB	Protein Kinase B
PKC	Protein Kinase C
PP2A	Protein Phosphatase 2A
PTEN	Phosphatase and Tensin Homologue deleted in chromosome 10
QPCR	Quantitative Polymerase Chain Reaction
Rb	Retinoblastoma susceptibility protein.
RICTOR	Rapamycin Insensitive Companion of mTOR
RING	Really Interesting New Gene
RTK	Receptor Tyrosine Kinase
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SHIP	SH2 domain containing inositol 5- phosphatase
TGF- β	Transforming Growth Factor β
TSC	Tuberous Sclerosis Complex
WHO	World Health Organization

Abstract.

Crosstalk between MDM2 and Akt signaling pathway in Oncogenesis

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor
of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

MDM2, the human homologue of the Mouse Double Minute 2 gene product, has been shown to be over-expressed in many cancers and to induce tumorigenesis. The role of MDM2 in oncogenesis was thought to be p53 dependent. However recent years have shown MDM2 to be a key player in a complex network of interactions that affect cell cycle, apoptosis, and tumorigenesis in a p53 independent manner. Here we report a novel p53 independent role for the multidimensional protein MDM2; its ability to induce phosphorylation of Akt at serine 473 residue. Transient and stable over-expression of MDM2 in cultured cell lines induces Akt phosphorylation. Silencing of MDM2 expression in cancer cells that over express MDM2 inhibits Akt phosphorylation suggesting endogenous MDM2 participates in Akt phosphorylation. Stable up-regulation of MDM2 expression reduced sensitivity of cells to chemotherapeutic drugs such as Etoposide,

Carboplatin or Paclitaxel. The domain of MDM2 responsible for drug resistance overlaps with the Akt phosphorylation domain. An inhibitor of Akt phosphorylation abrogated MDM2-mediated Akt phosphorylation and reduction of Etoposide sensitivity indicating that MDM2 reduces Etoposide sensitivity of cancer cells by activating the Akt phosphorylation. A PI-3 kinase inhibitor Wortmannin inhibits the ability of MDM2 to induce Akt phosphorylation and silencing of Rictor, a known kinase of Akt, does not hamper the ability of MDM2 to induce phosphorylation of Akt. MDM2-mediated Akt phosphorylation does not require p53, and the p53-interaction domain of MDM2 is dispensable for Akt phosphorylation. The presence of MDM2 enhances the Insulin like Growth Factor 1 mediated activation of Akt. Further cells harboring MDM2 show enhanced Interleukin 8(IL8) activation, which could be a possible mechanism of Akt activation. Downstream of Akt activation we show increased events that have been correlated with Akt activation like increased Bcl-2 levels increased processing of NF- κ B2, and GSK3 α/β phosphorylation among others. Our observation reveals a novel signaling function of MDM2 important for regulation of cell growth and chemotherapeutic sensitivity through Akt phosphorylation.

H. Chapter 1.Introduction

MDM2 is potentially oncogenic: Cancer is a group of diseases whose defining factor is uncontrolled growth of abnormal cells, which can metastasize- a process whereby the cells grow beyond their normal boundaries and spread to distant parts of the body. According to the WHO, in 2007 alone, Cancer accounted for nearly 7.9 million deaths (around 13% of all deaths) worldwide.

The human homologue of the murine double minute-2 gene product (MDM2) is weakly expressed in normal cells. But it is over-expressed in many human carcinomas, soft tissue sarcomas and other cancers, suggesting that the genetic alteration may be an important contributor to oncogenesis and disease progression(1-15). Over-expression of MDM2 has been shown to enhance tumorigenic potential of murine cells(16,17). Targeted tissue specific expression of MDM2 in transgenic mice causes polyploidy(18) . Over-expression of MDM2 in breast epithelial cells inhibits normal development of mammary gland (18)and targeted over-expression in the basal layer of epidermis increases papilloma formation by chemical carcinogens(19). Amplification of *mdm2* gene, Over-expression of *mdm2* mRNA(4,10,20,21) and enhanced translation of the *mdm2* mRNA (11,22,23)are proposed mechanisms of MDM2 over-expression.

Frequency of MDM2 amplification: MDM2 is found in very low amounts in normal cells. However the overall frequency of MDM2 amplification in both benign and malignant tumors is about 7%. 20% of all soft tissue tumors which include Ewing's sarcoma(13), leiomyosarcomas(24-26), lipomas(25,27), liposarcomas, malignant fibrous

histiocytomas(20,24,27-29), malignant schwannomas(20,24,25,27,30) and other sarcomas such as rhabdomyosarcomas(31) have MDM2 amplication. 16% of all osteosarcomas have MDM2 amplification(12,20,24,27).Roughly 10% of all gliomas over express MDM2(8,32-34). MDM2 over-expression has also been reported in about 13% of lung carcinomas, breast cancers and testicular cancers(15).

MDM2 as a prognostic marker: Though over-expression of MDM2 is a common event in cancer, as a prognostic marker it presents a paradox. Over-expression of MDM2 correlates with poor prognosis in sarcomas, breast cancers and lymphomas(32,35) and with good prognosis in lung carcinoma, malignant melanoma and ovarian cancer(3,36-39). This is further complicated by the co-occurrence or lack of p53 expression. Among soft tissue sarcomas, it was noticed that cases with mutant p53 and MDM2 expression had the worst prognosis, suggesting a Wild Type (WT) p53 independent oncogenic role for MDM2. Patients lacking both p53 and MDM2 had the best prognosis. In gliomas, where MDM2 is found over-expressed in nearly 10% of all cases, p53 mutations rarely co-occur with MDM2 over-expression. And in pediatric acute lymphoblastic leukemia, loss of WTp53 is a very rare event, though MDM2 mRNA over-expression has been shown to correlate with poor prognosis(32). Therefore tissue specific, differentiation specific and growth specific pressures exist, to which the different tumor subsets respond in different ways.

MDM2 and p53: The mdm2 gene product was originally detected in a complex with p53 (40). This association has been the most studied and the interaction has been found to be involved in an auto regulatory feedback loop. MDM2 can recognize the transactivation domain of p53 and thus inactivate the p53 mediated transcriptional activation. This

interaction has been shown both in vivo and in vitro. MDM2 is an E3 ubiquitin ligase and following binding to p53, it can target p53 for ubiquitination. For the ubiquitination function, MDM2 employs the RING finger domain for mono ubiquitination and the p300 interaction domain for poly ubiquitination. It is important to note the mutants of MDM2 lacking the ubiquitin ligase domain can bind to p53 and inhibit p53 mediated transcriptional activation(41-44). Therefore inhibition of transactivation and degradation of p53 are probably two distinct ways to regulate p53. (20, 40, 45-51). Also MDM2 ubiquitinates itself and thus regulates its expression.(52) MDM2 can be transcriptionally activated by p53 (20,40,45-51). The MDM2 gene has two distinct promoters and p53 mediated activation from the P2 promoter yield a distinct splice transcript differing in the 5' untranslated region from the P1 promoter product. (53).

MDM2 interactions: MDM2 interacts with a gamut of proteins in vivo. Consistent with its oncogenic potential, MDM2 interacts with several known growth suppressors, including p53, the p53 family protein p73(54), the retinoblastoma susceptibility gene product Rb(55) and growth suppressor p14 ARF (56,57)and p19(58,59). MDM2 has ben shown to interact with histones and histone deacetylases(60). It has been shown to interact with ribosomal proteins(54). Other MDM2 partners include, the p53 coactivator p300(61), the p53 co-regulator MDMX(62), the transcription factors E2F1/DP1(63) and SP1(64),mutant p53(65), cell fate protein NUMB(66) and the ribosomal protein L5(54). Mayo et al showed that after growth factor stimulation MDM2 interacts with Akt and gets phosphorylated, thus linking the mitogenic signaling pathway and MDM2(67). Later studies by various groups showed that MDM2 was able to ubiquitinate components of the mitogenic

signaling pathway including IGF1-R(68), β Arrestins(69) and G protein coupled Receptor Kinase 2(GRK2)(70).

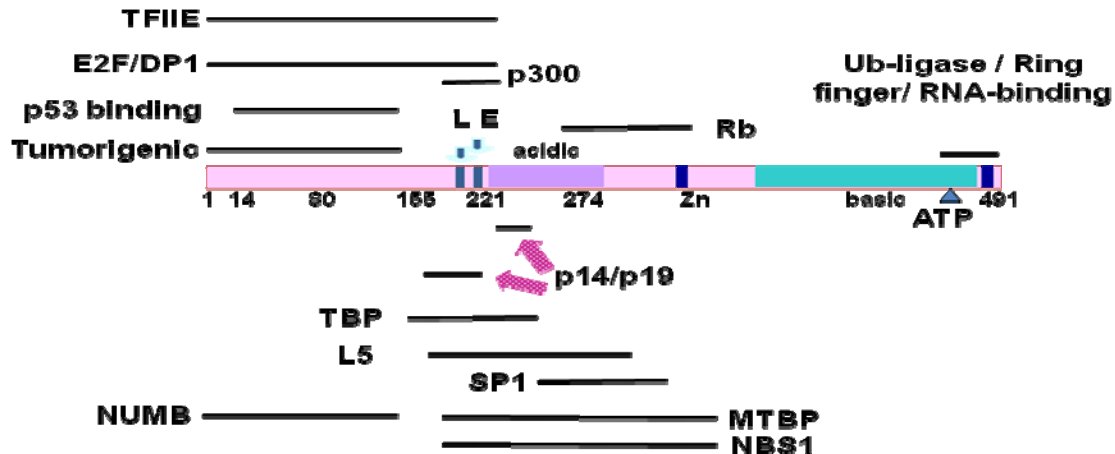


Figure 1. MDM2 interacting proteins: The domains of MDM2 where it binds to its interacting partners are shown by the black lines. MDM2 has many interacting partners in the cell, involved in a wide range of functions. These include cell cycle, tumorigenesis, differentiation, DNA synthesis, DNA repair, RNA biosynthesis, Transcription among others.

P53 independent activities of MDM2: Though most of the initial studies on MDM2 extensively characterized MDM2 as a p53 regulator, there is increasing evidence of p53 independent activities of MDM2. Transgenic mice with full length MDM2 are predisposed to spontaneous tumor formation in a p53^{-/-} background and have a high incidence of lymphoma and sarcoma.(71) The interactions of MDM2 with factors which function as regulators in processes like transformation, cell cycle control, DNA synthesis, RNA biosynthesis, transcription, mitogenic signaling pathways can happen in a p53 null environment(54).

MDM2 has been shown to confer growth advantage in cells devoid of p53 and pRb(72). Increased MDM2 expression has been shown to correlate with TGF- β resistance in human breast tumor cells independent of p53 status contributing to tumorigenesis(73). Amplification of MDM2 was found to interfere with MyoD activity in rhabdomyosarcoma, thus inhibiting overt muscle differentiation independent of p53(74). Numb, a cell fate protein involved in neural cell differentiation is a binding partner of MDM2 and gets ubiquitinated by MDM2(75). MDM2 can form a complex with pRb in vivo and blocks pRb mediated G1 arrest(55). MDM2 can also interact with E2F/DP1 transcription factor involved in S-phase progression(63). Yeast 2-hybrid screens and in vivo co-immunoprecipitations have shown MDM2 to interact with DNA Polymerase ϵ (76). MDM2 has also been shown to bind to the NBS1 protein and inhibit double strand break repair(77). The central domain of MDM2 was shown to interact with L5, a component of the large ribosomal subunit independent of p53(78). MDM2's role in transcription can be evidenced by its interaction with the TATA Binding Protein(79), Transcription Activating Factor II D(54), transcriptional co-activator p300(61) and by its ability to induce expression of p65(80). Recent years have shown the role of MDM2 in the rapid turnover of surface receptors in response to mitogenic signal activation. MDM2 has been shown by various groups to bind and ubiquitinate the Androgen Receptor(81), and the Insulin like Growth Factor-1 Receptor(IGF-1 R)(68) and also key regulators like β arrestins (69) and G Protein Coupled Receptor Kinase(GRK2) (70) among others.

MDM2 interacts with Efl α : In order to further study the p53 independent activities of MDM2, our lab carried out a mass spectrometric analysis of MDM2 bound proteins in

H1299, non small cell lung cancer cell line which was devoid of functional p53. Among the various proteins identified as potential MDM2 binding partners was the translational Elongation Factor (EF1 α). This result was further confirmed by immunoprecipitation experiments(82). EF1 α was shown by various groups to activate the Phosphatidylinositol 4 kinase(83), which in turn activated the Phosphatidylinositol 3 Kinase pathway resulting in activated Protein kinase B or Akt. This was shown to increase actin remodeling and result in increased motility(84).

MDM2 interacts with Akt: It has been reported that MDM2 and Akt interact in the cytoplasm of the cell. This interaction leads to the phosphorylation of MDM2 at residues serine 166 and serine 186(67,85). Later Milne et al showed that amino acids 108- 200 of MDM2 were required for the complete phosphorylation of MDM2 by Akt and that a deletion mutant of MDM2 harboring amino acids 196-282 was phosphorylated, but to a lesser extent(86). This seems to suggest that there may be two domains of MDM2 where Akt could bind to and phosphorylate it. Akt mediated MDM2 phosphorylation was shown to be required for the nuclear entry of MDM2. This facilitated the interaction of MDM2 and p53 and subsequent degradation of p53 after ubiquitination of p53 and its nuclear export.

The PI3K- Akt Pathway: The Phosphatidylinositol 3'-kinase (PI3K)-Akt signaling pathway regulates fundamental cellular functions such as transcription, translation, proliferation, growth and survival. Akt or protein kinase B is a serine/threonine kinase and the most important effector molecule in the pathway. The deregulation of the pathway has

been associated with the development of diseases such as cancer, diabetes mellitus and autoimmunity(87-92).

PI3K is responsible for the phosphorylation of the 3 position of the inositol ring of $\text{PI}(4,5)\text{P}_2$, to generate $\text{PI}(3,4,5)\text{P}_3$, a potent second messenger required for survival signaling and insulin action(93-95). PI3K is composed of two subunits: a catalytic subunit p110 and a regulatory subunit p85, which is activated by receptors with protein tyrosine kinase activity (Receptor Tyrosine Kinase, RTK) and by G protein coupled receptors (GPCR). Within seconds, activated PI3K converts plasma membrane lipid $\text{PI}(4,5)\text{P}_2$ to $\text{PI}(3,4,5)\text{P}_3$ (93-96). This prompts the translocation of Akt to the inner side of plasma membrane and binding to the phospholipids.(93, 95) This results in a conformational change in Akt and subsequent exposure of its two main phosphorylation sites; Threonine 308 and Serine 473. Activation of $\text{PI}(3,4,5)\text{P}_3$ also allows binding of Protein serine/threonine kinase 3'phosphoinositide Dependant Kinase (PDK1) to $\text{PI}(3,4,5)\text{P}_3$ and to Akt. This leads to the phosphorylation of Akt at Threonine 308 residue. For the full activation of Akt, the serine 473 residue needs to get phosphorylated(89,94,95,97,98). Many players have been identified or suspected to cause the phosphorylation at this residue. Rictor(99), Integrin Linked Kinase(ILK)(100-102) and Akt itself(103) are a few of these. Akt also gets activated in response to oxidative and osmotic stress, irradiation and treatment of cells to chemotherapeutic agents and ischemic shock(90). Recently it has also been shown that phosphorylation of Akt at serine 473 can occur in response to DNA damage and DNA dependant Protein Kinase (DNA-PK) was responsible for the event(104).

Regulation of the Akt phosphorylation event is achieved by phosphatases which act at various stages along the PI3K/Akt pathway. The Phosphatase and Tensin Homologue deleted on chromosome 10 (PTEN) is a phosphatase that can reverse the conversion of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ (105). $PI(3,4,5)P_3$ can be converted to $PI(3,4)P_2$ by the SH2 domain-containing inositol 5-phosphatase (SHIP)(98) The phosphatases in the PHLPP family, PHLPP1 and PHLPP2 have been shown to directly de-phosphorylate, and therefore inactivate, distinct Akt isoforms(106,107). Phosphatase 2A (PP2A) is another enzyme involved in the dephosphorylation of Akt. (108) Another protein, the Carboxy Terminal Modulating Protein (CTMP) binds to the C terminus of Akt, where it dephosphorylates Akt(109).

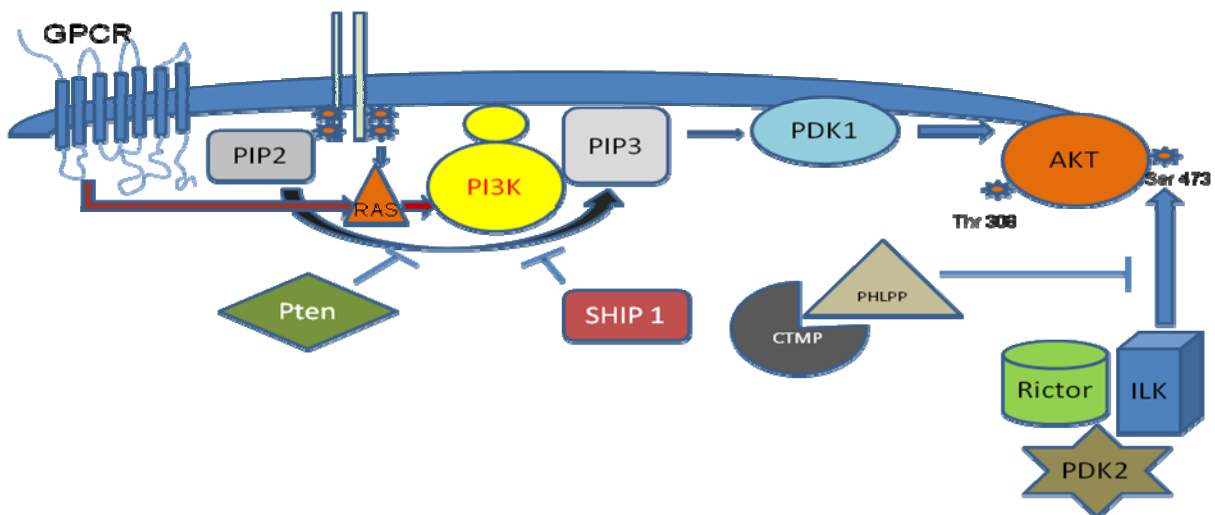


Figure 2. The PI3K-Akt pathway regulation: The binding of growth factors to their receptor tyrosine kinase (RTK) or the G Protein Coupled Receptors (GPCR) stimulate the phosphorylation of PI3K. Activated PI3K converts PIP2 to PIP3, whereas PTEN and SHIP reverse this reaction. Akt translocates to the membrane and gets phosphorylated at Threonine 308 by PDK1. This is followed by phosphorylation at Serine 473 by kinases such as Rictor, ILK etc. Dephosphorylation at Serine 473 can be achieved by enzymes such as PHLPP and proteins like CTMP.

Functions of Akt: Activated Akt plays vital roles in cell processes such as survival, proliferation, motility, resistance to chemotherapeutic agents, cell cycle progression and glucose metabolism by phosphorylating various downstream targets(89,90).

Phosphorylation of Bad by Akt promotes survival by preventing Bad from binding and inhibiting anti apoptotic BCL-x_L and from altering the mitochondrial membrane potential(110,111) Akt has also been shown to increase antiapoptotic BCL-x_L through activation of NFκB(112) and Bcl2 by increasing CREB binding to the promotor of Bcl2(113). The caspase cascade is also a target for Akt activity. Akt has been shown to phosphorylate and inhibit the activity of caspase 9(114). Akt phosphorylation of FKHL1, a forkhead transcription factor allows binding to 14-3-3 proteins and subsequent retention in the cytoplasm(115-117). This prevents expression of its transcription targets like Fas ligand, Bim and IGFBP1 that in turn promote apoptosis(89,118-120). Phosphorylation and in-activation of IκB kinase α (IKKα) promotes the activation of both the canonical and non canonical NFκB pathways which play a critical role in cell survival, motility and immunity(121-123). Akt promotes cell cycle progression by regulating cyclin D function. This is accomplished by phosphorylation of p27^{Kip1} and p21^{Waf1} by Akt(124,125). This prevents them from interacting with the respective cyclins. MDM2 has also been shown to be a substrate of Akt phosphorylation. Phosphorylation of MDM2 by Akt has been shown to be required for the nuclear entry of MDM2 into the nucleus and the steps leading to its binding to and degradation of p53(67,85,86). One of the earliest substrates of Akt was shown to be Glycogen synthase kinase 3 (GSK3).GSK-3 is known for phosphorylating and thus inactivating glycogen synthase, proving a role for Akt in the insulin signaling

pathway and metabolism(126-128). Akt also plays a role in protein synthesis by increasing the phosphorylation of mamalian Target of Rapamycin (mTOR) through the phosphorylation of Tuberous Sclerosis Complex (TSC) upstream of mTOR.(129). This promotes the activation of p70 ribosomal protein S6 kinase (p70S6K1) and inhibition of eukaryotic initiation factor 4E-binding protein1 (4EBP1), thus promoting protein synthesis(129-132).

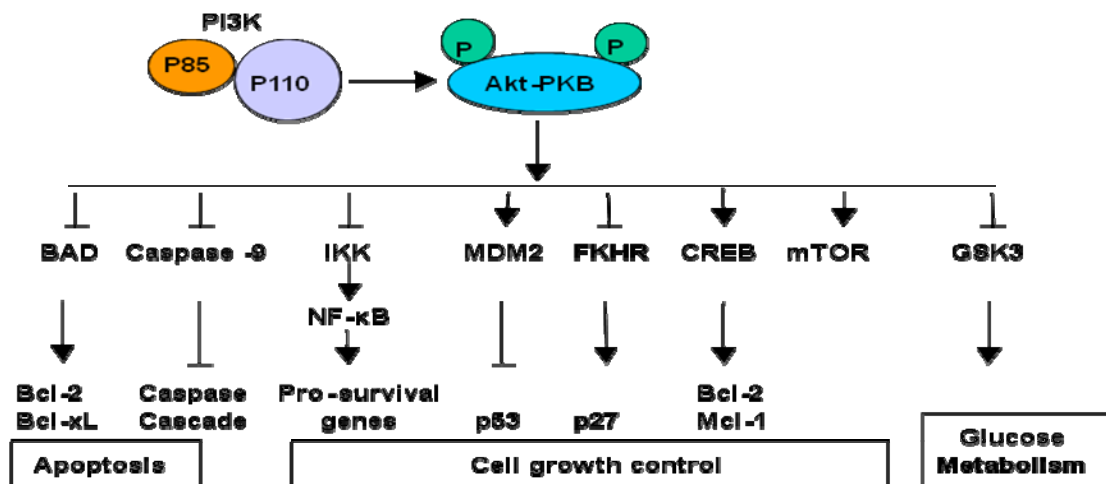


Figure3. Functions of Akt: Activation of Akt promotes phosphorylation and loss of function of pro apoptotic proteins including BAD and Caspase9 and increase in anti apoptotic proteins like Bcl-2, Bcl-x_L. Akt also phosphorylated MDM2 which reduces p53 levels and therefore apoptosis. Akt inhibits cell cycle arrest by phosphorylation of cdk inhibitors p27and p21 and by increasing the activity of the NFκB pathway after phosphorylation of IKKα. By phosphorylation of GSK3 Akt prevents phosphorylation of targets like Glycogen synthase, thus playing a role in the insulin pathway. Protein synthesis is enhanced by Akt by phosphorylation of mTOR and pathway members, p70S6K and 4EBP1.

I. Chapter 2. Hypothesis

a. Major Hypothesis.

The goal of cancer biology is the identification of biomarkers which can predict the disease or the therapeutic response. Cancer is a result of mutation of more than one gene and hence deregulation of more than one pathway. It is therefore not possible to attribute the distinct cancer phenotypes to mutation in one specific biomarker or another. MDM2 is amplified in many different cancer subtypes. However its value as a prognostic marker remains unclear, depending on tumor type, tissue of origin and other factors. It presents a paradox; over-expression of MDM2 correlates with poor prognosis in some tumors and good prognosis in others. Also over-expression of MDM2 has been shown to offer resistance to various chemotherapeutic agents. Only a more profound understanding of the biology underlying MDM2 expression and consequence of its expression in different cancer subtypes can address the drawback of most translational studies where a reductionist approach of tailoring treatment based on the expression of MDM2 alone is followed. Therefore the crux of the study is to identify what markers get expressed or repressed as a result of MDM2 expression in different cancer subtypes and thus tailor treatment strategies for different cancer subtypes.

The major hypothesis of this work involves deciphering whether p53 independent over-expression of MDM2 leads to activation of Akt, thus identifying a novel pathway. More importantly the study focused if preventing the activation of Akt by MDM2 could render drug sensitivity to MDM2 over-expressing cells. This hypothesis was based on previous observations in our laboratory and by others. Our laboratory identified that MDM2

interacts with the Eukaryotic Translational Elongation Factor EF1 α . EF1 α was shown by various groups to activate the Phosphatidylinositol 4 kinase, which in turn activated the Phosphatidylinositol 3 Kinase pathway resulting in activated Protein Kinase B or Akt. This was shown to increase actin remodeling and results in increased motility. Therefore the fact that MDM2 and EF1 α interact led us to ask whether the interaction could lead to the Akt activation.

Further, other groups showed the interaction of MDM2 and Akt in the cytoplasm, and this interaction being required by MDM2 for entry into the nucleus. This observation prompted us to raise the question on what would happen if there was an enhanced level of MDM2. Since MDM2 over-expression has been shown to increase the tumorigenicity of cells, mainly by entering the nucleus and degrading p53 and MDM2 required phosphorylated Akt to allow its nuclear entry, it should seem implicit that for the higher level of MDM2, there should be higher levels of activated Akt. This also fell in place with our observation that MDM2 interacted with EF1 α and that EF1 α was shown to activate the Akt pathway. Since the interaction of MDM2 and EF1 α was a p53 independent interaction, we asked whether MDM2 could activate the Akt pathway independent of p53.

Since the Akt pathway activation has been correlated to resistance to various chemotherapeutic agents; we propose that MDM2 over-expression in cells could render resistance to chemotherapeutic agents by activating the Akt pathway. Therefore in subsets of tumors where both MDM2 and phosphorylated Akt are co-expressed, agents targeting the Akt pathway and thus negating the resistance offered by MDM2 could be plausible treatment strategy to investigate.

b. Specific Aims.

The following are the specific aims:

1. A. To investigate if MDM2 can induce Akt phosphorylation in a p53 independent manner.

B. To examine whether MDM2 induced Akt phosphorylation can render the cells resistant to Etoposide treatment.
2. To decipher the mechanism by which MDM2 can activate the Akt pathway
3. To determine the events downstream of MDM2 mediated Akt phosphorylation

J. Chapter 3

Human Oncoprotein MDM2 enhances Akt phosphorylation at Serine 473 residue, reducing sensitivity of cancer cells to chemotherapeutic drugs.

The manuscript for the work presented in this chapter is currently under preparation. Quantitative RT PCR on WI38 cells was performed by Lathika Mohanraj.

a. Introduction.

MDM2 has been shown to be oncogenic in a p53 independent environment(54,71). Our laboratory showed that MDM2 can interact with EF1 α (82). Since EF1 α is known to up regulate PI4Kinase(83) and thereby activate the Akt pathway(84), we wanted to test if MDM2 over-expression could result in Akt activation. Also it has been shown by other laboratories that MDM2 requires phosphorylation by Akt for its nuclear entry, a mechanism by which MDM2 interacts with p53 and degrades it(67,85,86). This has been one of the classical mechanisms by which MDM2 induces tumorigenesis. Therefore it would be implied that in the presence of more MDM2, Akt phosphorylation would be enhanced to facilitate MDM2's nuclear entry.

MDM2 has been suggested as an important target for cancer therapy(133). Various groups have shown the use of polypeptides(134,135), antibodies(136), small molecule inhibitors(137) and antisense approach against MDM2(138-140) as treatment strategies. However these studies have raised more questions than answers. Over-expression of MDM2 has been shown to offer resistance to various chemotherapeutic agents in some cases and in others, sensitization to drugs(141,142). For example, one group evaluated the response of 107 patients undergoing surgical resection and adjuvant chemotherapy

for esophageal squamous cell carcinoma and found that MDM2 expression correlated with shorter survival for patients undergoing post operative chemo- or radiotherapy. In stark contrast, a study on advanced breast cancer found that high levels of MDM2 corresponded to a better response to treatment and longer time to progression. To make matters more complicated, other studies have indicated no correlation between MDM2 expression and response to chemotherapy(141). As of 2005, there were no anti-MDM2 drugs in clinical trials and only agents blocking expression of relevant molecules were being tested(141).

Numerous groups have reported that the administration of chemotherapeutic agents results in the modulation of the PI3K/Akt pathway (143-150). Significantly, the pathway has been shown to be activated in response to treatment by chemotherapeutic agents, which fall under the category of anthracyclins(143,144), topoisomerase inhibitors(147), microtubule agents(149) and DNA damaging agents(148). The activation of the PI3K/Akt pathway by these agents has also been shown to correlate with drug resistance(143,144,147-149). Since we hypothesize that MDM2 enhances Akt phosphorylation, this could be a mechanism of MDM2 mediated drug resistance.

b. Experimental Results.

MDM2 induces AKT phosphorylation at Serine 473. To check if MDM2 expression results in the induction of Akt phosphorylation, we chose to transfect non small cell lung cancer H1299 (p53-null) or normal diploid WI38 (p53 WT) cells. The cells were chosen to take into account the requirement of p53 and also to find out if this phenomenon could be seen in normal cells. To transiently over express MDM2, we nucleofected the cells

with MDM2 expression plasmids using a nucleofector and reagents (Amaxa), according to the supplier's protocol. Cell extracts were subjected to Western blot analysis to determine the levels of phosphorylated Akt, Akt and MDM2. Western blot analysis using an antibody against Akt phosphorylated at Ser 473 indicated that extracts prepared from H1299 or WI38 cells transfected with the MDM2 expression plasmid contain significantly higher levels of phosphorylated Akt than extracts prepared from vector transfected cells (Figure 4A, 4B). These data show that over-expression of MDM2 enhances Akt phosphorylation in normal diploid or transformed cells in the presence or absence of p53, and therefore Akt phosphorylating function of MDM2 is p53-independent.

We tested whether stable over-expression of MDM2 in H1299 cells elevates the levels of phosphorylated Akt. For generating H1299 stable transfectants, H1299 cells nucleofected with a vector or a MDM2 expression plasmid harboring a neomycin resistance gene were selected for neomycin resistant colonies. Pooled colonies were maintained in neomycin containing media. Cell protein extracts were subjected to Western blot analysis to determine the levels of phosphorylated Akt, Akt and MDM2. The results (Figure 4C) show that stable expression of MDM2 induces phosphorylation of Akt in H1299 cells.

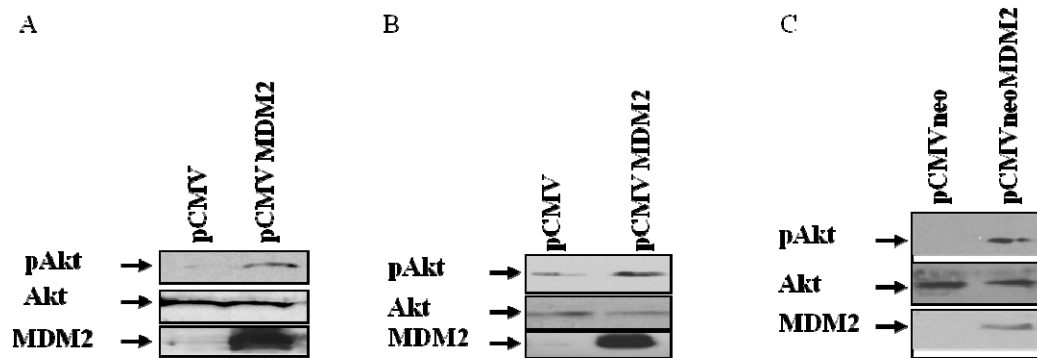


Figure 4. MDM2 Induced Akt Phosphorylation at serine 473: Western blot analysis of extracts prepared from H1299 (A, C) or WI38 (B) cells transiently (A, B) or stably (C) nucleofected with MDM2 expression plasmids (pCMV MDM2, pCMV neo MDM2) or vector plasmids (pCMV, pCMV neo). p-Akt was identified with an antibody specific for Akt phosphorylated at Ser 473 (#9271 rabbit polyclonal, Cell Signaling); Akt (sc8312 rabbit polyclonal, Santa Cruz), MDM2 (AB1, Oncogene Science) and actin antibodies (AC-15, Sigma) were used to identify Akt, MDM2 and actin. Migration of the control MDM2, p-Akt and Akt bands are shown by arrows.

Silencing of MDM2 expression reduces endogenous Akt phosphorylation at serine

473: We next determined whether endogenous MDM2 has a role in enhancing Akt phosphorylation. An osteosarcoma cell line, SJSA-1, which harbors WTp53 and over expresses MDM2(151) was transfected with a short interfering (si) RNA against MDM2. Furthermore, three plasmids harboring a puromycin resistance gene were constructed two of which expressed two different shRNA against MDM2 and one expressed shRNA against a non-specific luciferase gene. The plasmids were nucleofected in SJSA-1 cells. Puromycin resistant colonies were generated and pooled. Extracts prepared from siRNA-transfected cells or puromycin resistant pooled cell colonies were subjected to Western blot analysis to determine levels of MDM2 and phosphorylated Akt. The results show that transfection with MDM2 siRNA diminished MDM2 expression remarkably with a

corresponding decrease in the levels of phosphorylated Akt compared to control siRNA transfected SJSA-1 cells (Figure 5A). Similarly, MDM2 shRNA expressing plasmids inhibited MDM2 expression and Akt phosphorylation compared to plasmid expressing luciferase shRNA (Figure 5B). These data show that diminished MDM2 expression causes reduction in Akt phosphorylation, indicating that endogenously overexpressed MDM2 in SJSA-1 cells enhances Akt phosphorylation.

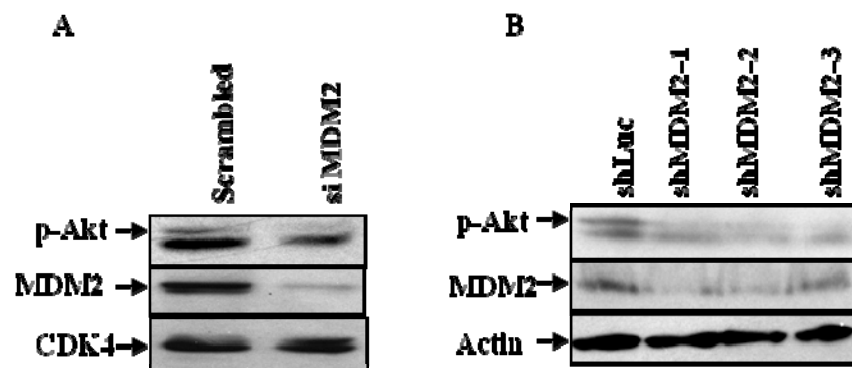


Figure 5. Silencing of endogenous MDM2 expression in SJSA-1 cells reduces Akt phosphorylation: Western blot analysis of extracts prepared from SJSA-1 cells (A) transiently transfected with MDM2 siRNA or non specific scrambled RNA and (B) stably transfected with plasmids expressing MDM2 shRNA (shMDM2-1, 2 or 3) or luciferase shRNA (shLuc). Migration of MDM2, p-Akt CDK4 and actin are indicated.

MDM2 induces drug resistance in cancer cells: Various groups have reported the modulation of the PI3K/Akt pathway in response to administration of chemotherapeutic agents. The pathway has been shown to be activated in response to treatment by chemotherapeutic agents, which are used in clinical setting. The activation of the PI3K/Akt pathway by these agents has also been shown to correlate with drug resistance(143-150).

Since MDM2 over-expression led to activation of Akt in our experiments, we decided to determine whether the over-expression of MDM2 led to resistance to the chemotherapeutic agents, which have been shown to have a decreased efficiency when the Akt pathway is activated(143,144,147-149). We chose one example from three classes of chemotherapeutic agents:

Topoisomerase inhibitors: Etoposide, which has been shown to inhibit topoisomerase II. Etoposide blocks the ligation step of the DNA replication, generating single and double stranded breaks that harm the integrity of the genome. Introduction of these breaks subsequently leads to apoptosis and cell death(152).

Microtubule poison: Paclitaxel; works by interfering with normal microtubule breakdown during cell division. Paclitaxel has been shown to a potent mitotic inhibitor(153).

DNA alkylating agents: Carboplatin; which crosslinks DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible(154).

To test for drug resistance, we used equal numbers of the control vector and MDM2 over-expressing H1299 cells that we generated as pooled clones and treated them with Etoposide, Paclitaxel or Carboplatin (and appropriate vehicles) and the drug response was analyzed by colony formation. Drug sensitivity was determined at increasing Etoposide or Paclitaxel concentration and 1.5 μ M concentration of Carboplatin. The concentrations were chosen to reflect the serum levels of these drugs, when used in therapy. Our data show that p53-null H1299 cells stably over-expressing MDM2 generated 2- to 5- fold higher number

of colonies than vector transfected cells after treatment with Etoposide at all concentration ranges (Figure 6A). Similar results were observed with Paclitaxel (Figure 6B) and Carboplatin (Figure 6C). These results suggest that MDM2 can enhance tolerance of cancer cells to chemotherapeutic agents.

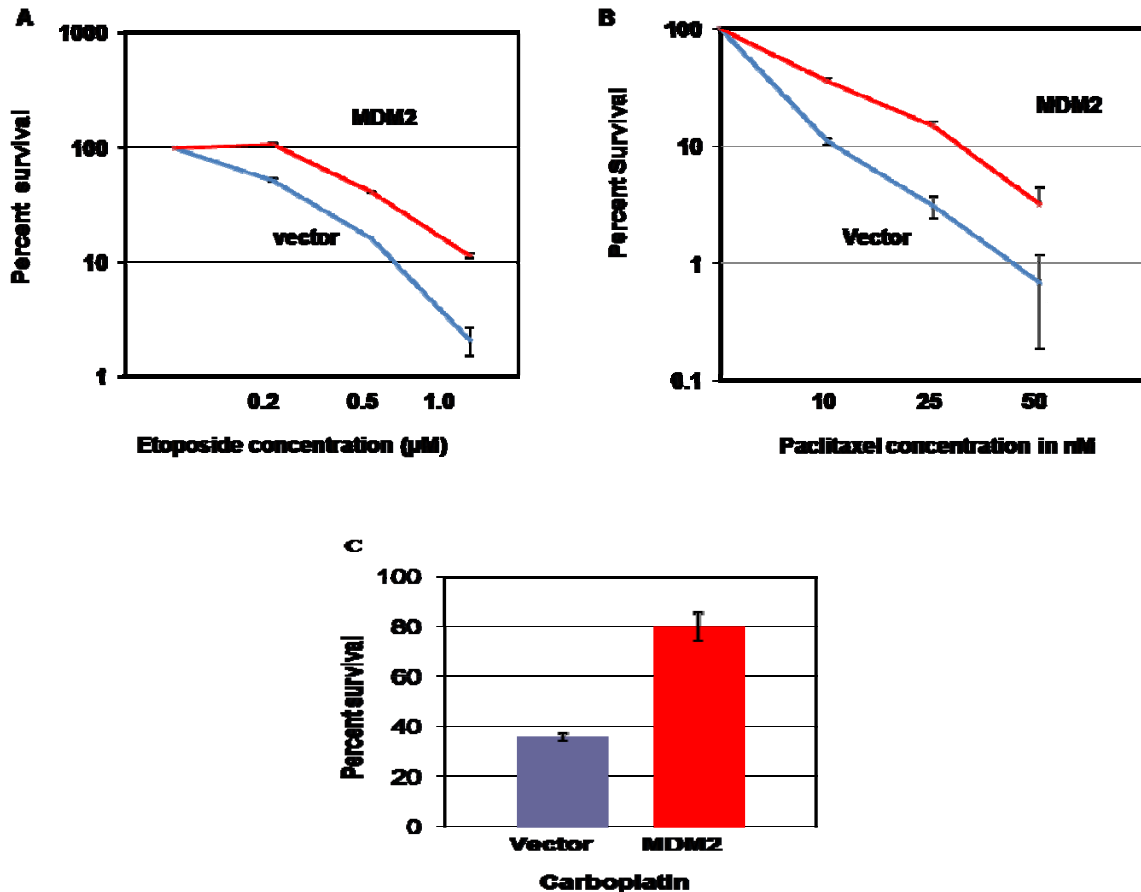


Figure 6. Over-expression of MDM2 reduces sensitivity of H1299 cells to Etoposide, Paclitaxel and Carboplatin. Colony assays were performed using H1299 cells stably transfected with vector or MDM2 expression plasmid after treatment with (A) increasing concentration of Etoposide, (B) increasing concentration of Paclitaxel or (C) 1.5 μM Carboplatin. Percent survival was calculated considering number of colonies generated in the vehicle treated plates to be 100%. The results are shown by (A,B) semi logarithmic curves plotting percent survival against increasing drug concentration, or (C) bar graphs to compare percent survival of H1299 stable transfectants after treatment with Carboplatin. Each experiment was performed in triplicate. Error bars representing standard deviations of the colony numbers are shown.

Silencing of MDM2 expression increases sensitivity of cancer cells to Etoposide: To determine if endogenous MDM2 plays a role in the resistance to chemotherapeutic agents, we used the MDM2-2 short hairpin expressing SJSA-1 cells (Figure 5B) which were stably maintained pooled clones. The effect of siRNA wears off after about 48 hours. Therefore, we used stable shRNA pooled clones of SJSA-1 to find out what happens when there is sustained silencing of endogenous MDM2. Equal numbers of SJSA-1 cells stably expressing MDM2 shRNA or luciferase shRNA were either treated with 1 μ M Etoposide or increasing concentrations of Paclitaxel (and the appropriate vehicles). Response of these cells to Etoposide and Paclitaxel was determined by colony assay. Our data show that SJSA-1 cells stably over-expressing MDM2 shRNA reproducibly generated 2-fold less number of colonies than luciferase shRNA expressing cells after treatment with Etoposide (Figure 7C) and 2-4 fold less number of colonies than luciferase shRNA expressing cells after treatment with increasing concentrations of Paclitaxel, showing that reduction in the levels of endogenous MDM2 enhances susceptibility of the cells to Etoposide and Paclitaxel. These data indicate that endogenously over-expressed MDM2 contributes to the tolerance of the cancer cells to Etoposide and Paclitaxel.

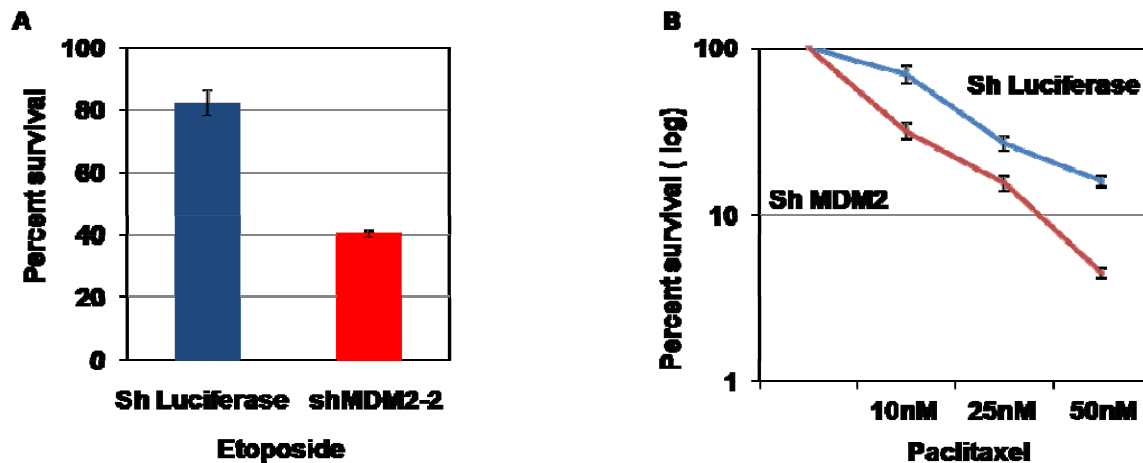


Figure7. Silencing of MDM2 expression increases sensitivity of cancer cells to chemotherapeutic agents: Colony assays were performed in SJSA-1 cells, stably transfected with Sh RNA against MDM2 or Luciferase expression plasmid after treatment with (A) 1 μ M of Etoposide or (B) increasing concentration of Paclitaxel. Percent survival was calculated considering number of colonies generated in the vehicle treated plates to be 100%. The results are shown by bar graphs (A) or logarithmic curves plotting percent survival against increasing drug concentration (B). Percent survival was calculated considering number of colonies generated in the vehicle treated plates to be 100%. Each experiment was performed in triplicate. Error bars are shown and represent standard deviations of the colony numbers.

Silencing of MDM2 expression increases sensitivity of normal diploid cells: Antisense approach to target MDM2 in cancer cells has been proposed by various groups to be a potential therapeutic strategy. The advantage antisense oligos are thought to have over other approaches like small molecule inhibitors (for eg: nutlin) is that it can target both p53 dependent and independent contribution of MDM2 towards oncogenesis. Synergistic studies using antisense oligos and chemotherapy have been shown to increase therapeutic effectiveness. However, not much is known on whether this approach may result in a similar cell death in normal cells; not just pertaining to the cancer environment, but also elsewhere in the body(142). This has been considered a potential drawback, since MDM2

expression in normal cells is extremely low and the balance is required for prevention of p53 activation in normal cells. To determine if endogenous MDM2 silencing in normal cells plays a role in the resistance to chemotherapeutic agents, we used MCF-10A, which is a non-tumorigenic epithelial cell line which is a surrogate for normal epithelial cells(156) and WI38, diploid lung fibroblasts. We used a lentiviral approach to silence MDM2 in these cells. In short, the plasmids expressing the short hairpin RNA against MDM2 were packaged inside a lentivirus system and used to infect the plated cells. This ensured a more efficient delivery of the plasmids into the cells and stable incorporation(157,158) (Figure 8B). Since the plating efficiency of WI38 cells was too low for clonogenic assays, following the infection equal numbers of the WI38 cells were plated in 6 cm dishes and treated with either Etoposide (1 μ M), Paclitaxel (25nM) or the respective vehicles and the cells were counted on a daily basis to check for the numbers using a coulter counter, which reflected the survival rate of the cells (Figure 8A). MCF-10A cells were infected with the lentivirus packaged with plasmids expressing short hairpin RNA targeting either non specific luciferase or MDM2 and equal number of cells were plated and treated with Etoposide (1 μ M), Paclitaxel (25nm) or appropriate vehicle. Response of the cells to drug was determined by clonogenic or colony assays. WI38 cells treated with shMDM2 were at least 2 times more susceptible to the treatment with Etoposide and Paclitaxel by day 7. By day 12, mere silencing of MDM2 in these cells, kills them. Also the cells show considerable death in response to drug treatment. MCF-10A cells also showed decreased colony formation when treated with the shRNA.

These results indicate that silencing MDM2 by antisense approach can lead to non targeted effects and potentially kill normal cells.

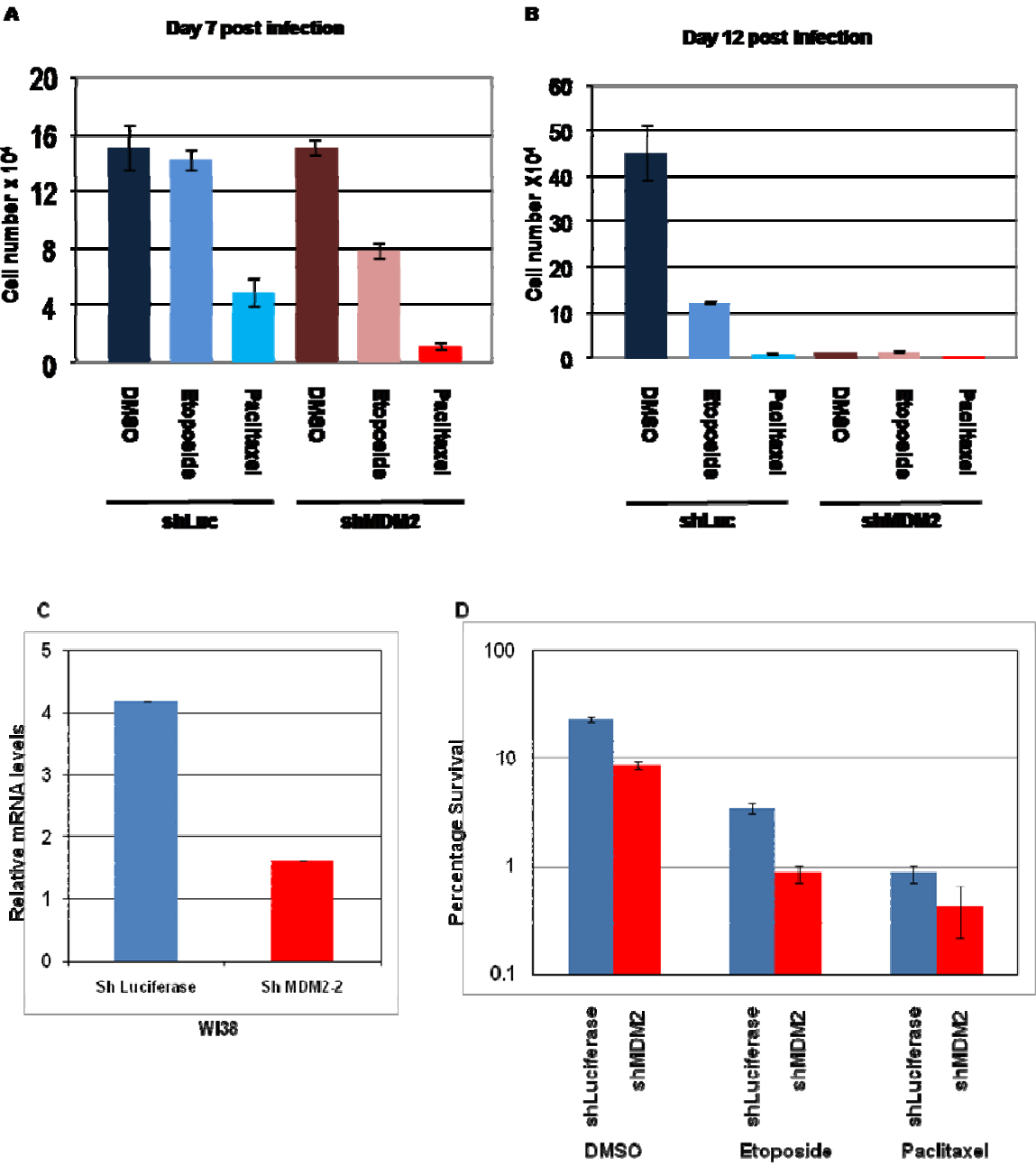


Figure8. Silencing of MDM2 expression increases sensitivity of normal cells to chemotherapeutic agents: Growth assay was performed using WI38 cells infected with lentivirus packaged with plasmid expressing shRNA against MDM2 or nonspecific luciferase using 1 μ M Etoposide, 25nM Paclitaxel or relevant vehicle. Cell numbers on Day 7(A) and Day 12(B) after drug treatment are shown by bar graphs. Knockdown of endogenous levels of MDM2 RNA by the shRNA was done in WI38 cells and checked by quantitative RT PCR. The relative fold levels are shown by bar graph (C). The RT PCR was done in technical triplicates. Error bars represent the standard deviations. Colony assays were performed using (D) MCF-10A cells infected with lentivirus packaged with non specific luciferase shRNA or shRNA against MDM2 using 1 μ M of Etoposide, 25nM of Paclitaxel or relevant vehicles. Percent survival was calculated considering number of colonies generated in the vehicle treated plates to be 100%. The results are shown by bar graphs. Each experiment (A, B and D) was performed in triplicate. Error bars indicate the standard deviations.

An inhibitor of Akt activation, Akt inhibitor III (SH-6) inhibits MDM2-mediated Akt phosphorylation at serine 473 and MDM2-mediated reduction of Etoposide

resistance: Since our data suggest that MDM2 activates Akt phosphorylation (Figure 4A-C), and reduces Etoposide sensitivity of cancer cells (Figures 6A, 7A), we determined if an inhibitor of Akt phosphorylation would abrogate ability of MDM2 to alter Etoposide sensitivity. For this purpose H1299 stable transfectants (vector and MDM2 expressing cells) were pretreated with Akt inhibitor SH-6 or the vehicle (DMSO) for four hours. Sh-6 is a phosphatidylinositol analog that inhibits the activation of Akt and select downstream substrates without decreasing phosphorylation of PDK-1 or other kinases downstream of Ras(159,160). Equal numbers of treated cells were then either plated to determine their sensitivity to Etoposide by colony development, or harvested to prepare cell extracts to determine Akt phosphorylation and MDM2 expression by Western blot analysis. The results of our colony assay show that while the MDM2 over-expressing H1299 stable transfectants pre-treated with DMSO generated approximately 3-fold higher number of

colonies than the corresponding vector-transfected stable transfectants after exposure to Etoposide, pre-treatment with SH-6 abrogated the ability of MDM2 to enhance the tolerance of H1299 stable transfectants to this drug (Figure 9A). The Akt inhibitor SH-6 inhibited Akt phosphorylation in both the stable transfectants as expected (Figure 9B). This data shows that MDM2-mediated Akt phosphorylation is required for its ability to enhance Etoposide tolerance of H1299 cells.

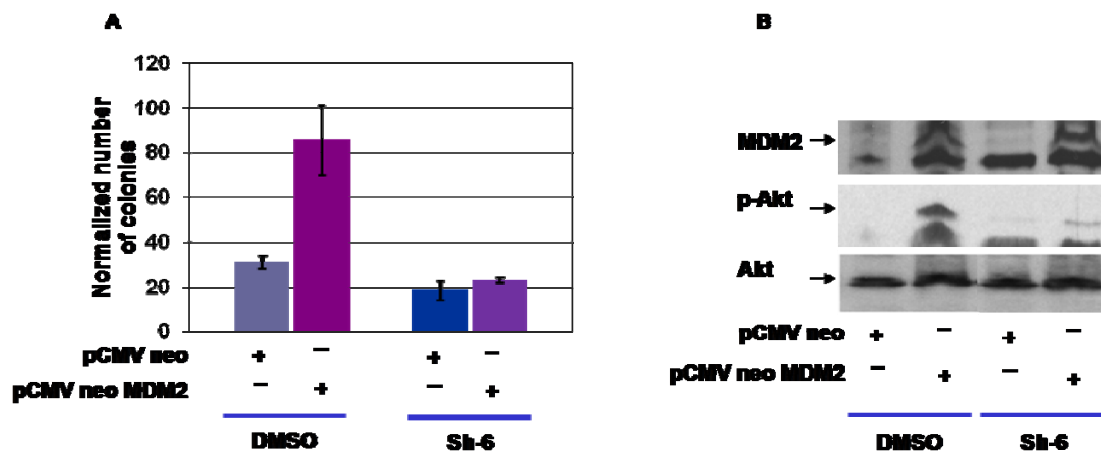


Figure9. Inhibition of Akt phosphorylation inhibits the ability of MDM2 to decrease Etoposide sensitivity. Left panel (A) shows a bar graph to compare number of colonies generated after treatment of H1299 stable transfectants (vector and MDM2 expressing) with 1.76 μ M SH-6 or DMSO followed by 6 μ M Etoposide and colony formation assay. The normalized number of colonies represents the ratio of colony numbers generated after treatment of cells with Etoposide to the number of colonies treated with vehicle. Experiments were done in triplicates and error bars indicating the standard deviations of the number of colonies formed are shown. The right panel (B) shows inhibition of MDM2-mediated Akt phosphorylation by SH-6. Akt and MDM2 expression are also indicated.

Increased Akt activation by MDM2 leads to enhanced proliferation rate: The Akt pathway activation has been shown to increase the proliferation of cells towards

oncogenesis(87-89). Therefore we did a proliferation assay to find out if MDM2 over-expressing cells had increased proliferation rates. Also we tested whether inhibiting the Akt pathway could render reduce the proliferation rate. H1299 stable transfectants (vector and MDM2 expressing cells) were pretreated with Akt inhibitor SH-6 or the vehicle (DMSO) for four hours. Equal numbers of treated and untreated cells were plated and allowed to grow. They were harvested and counted each day after plating and the growth rates plotted. Our results show that while MDM2 over-expressing cells had enhanced proliferation rate, it dropped to the rate of the control cells when the Akt phosphorylation in the cells was limited (Figure 10)

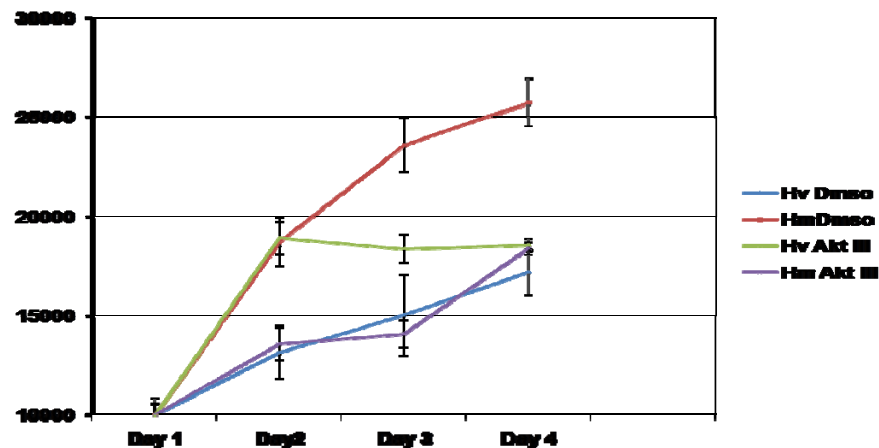


Figure10. Increased Akt activation by MDM2 leads to enhanced proliferation rate. H1299 stable transfectants (vector and MDM2 expressing) were pretreated with 1.76 μ M SH-6 or DMSO and plated. The cells were harvested each day and the numbers were counted using a coulter counter. They were normalized to the counts on day 1, which was used for plating efficiency. The experiments were done as technical triplicate and error bars representing the standard deviations of the counted cell numbers are shown.

Inhibition of Akt phosphorylation by SH-6 is not cytotoxic to normal cells: Next, we determined cytotoxicity of SH-6; whether pretreatment of SH-6 led to reduced number of colonies after plating. For this purpose WI38 were pretreated with Akt inhibitor SH-6 or the vehicle (DMSO) for four hours. Equal number of treated cells were either plated to determine their ability to form colonies, or harvested to prepare cell extracts to determine Akt phosphorylation by Western blot analysis. The results of our colony assay show that SH-6 treated cells did not have decreased colony formation ability when compared to the cells pretreated with the vehicle. (Figure 11A). The Akt inhibitor SH-6 inhibited Akt phosphorylation in WI38 as expected (Figure 11B). This data shows that using SH-6 at the concentration of 1.76 μ M is not cytotoxic to normal diploid cells.

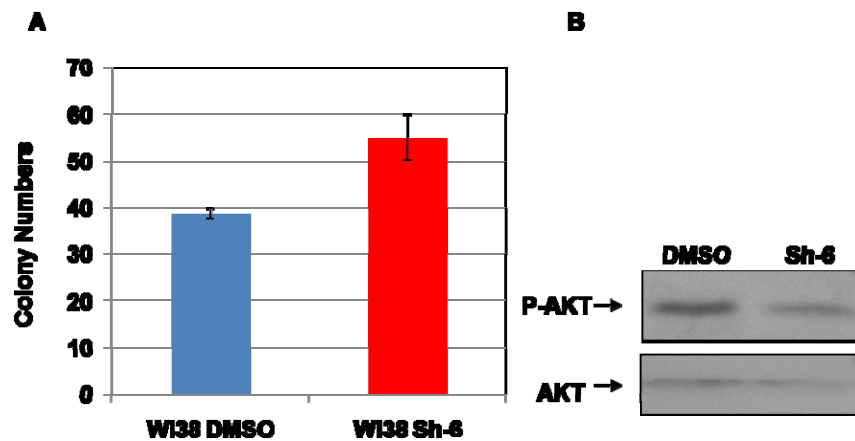


Figure11. Inhibition of Akt phosphorylation by SH-6 does not result in cell death. Left panel (A) shows a bar graph to compare number of colonies generated after treatment of WI38 with 1.76 μ M SH-6 or DMSO. The plating was done in triplicates and the error bars indicate the standard deviation of the number of colonies generated. The right panel (B) shows inhibition of Akt phosphorylation by SH-6. Total Akt is used as loading control and indicated.

c. Chapter Summary.

The human oncoprotein MDM2 interacts with the protein kinase B/ Akt that phosphorylates MDM2. Our observation shows that MDM2 enhances Akt phosphorylation at Ser 473. Stable over-expression of MDM2 expression reduces sensitivity of cells to chemotherapeutic drugs such as Etoposide, Carboplatin or Paclitaxel. MDM2 over-expression also leads to increased proliferation rate. Silencing of endogenous MDM2 expression inhibits Akt phosphorylation and enhances their sensitivity to Etoposide and Paclitaxel suggesting endogenous MDM2 participates in Akt phosphorylation and alteration of sensitivity to chemotherapeutic agents. An inhibitor of Akt phosphorylation abrogated MDM2-mediated Akt phosphorylation and reduced Etoposide sensitivity indicating that MDM2 reduces sensitivity of cancer cells by activating the Akt phosphorylation. Also inhibition of Akt phosphorylation resulted in decreased proliferation rate in MDM2 over-expressing cells. Our observation reveals a novel signaling function of MDM2, important for enhanced survival and proliferation, both characteristics of cancer cells.

K. Chapter 4.

Mechanism of MDM2 mediated activation of Protein Kinase B/Akt.

The manuscript for the work presented in this chapter is currently under preparation. The q-RT PCR for CXCL8 transcripts and migration assay was performed by Dr Andrew Yeudall.

a. Introduction.

In order to decipher a mechanism by which MDM2 expression resulted in activation of Akt, we decided to investigate the players involved in the PI3K/Akt pathway and what modulations were observed because of MDM2 over-expression. Akt is activated in response to a wide range of stimuli. These include growth factors, serum, survival factors and agonists which act through surface receptors. Besides these, Akt has been shown to be activated in response to various cellular insults such as oxidative and osmotic stress, irradiation and treatment of cells with chemotherapeutic agents and ischemic shock(89,90,93-95,104,161). Inhibition of cellular phosphatases also has been shown to activate Akt(161,162). It has been shown that the phosphorylation event in Akt follows it's docking to the plasma membrane and this requires the activation of the PI3K pathway(100,101,163,164). Though recent reports have shown PI3K independent mechanism of Akt activation(99), it still serves as the most well studied mechanism of Akt activation. Akt activation is a two step process; phosphorylation of Akt at Threonine 308 is followed by Serine 473 phosphorylation for full activation of the kinase. PDK1 is a well known kinase responsible for the phosphorylation of Akt at threonine 308(161). Though kinases such as Rictor(99), DNA-PK(104), ILK(100-102) have been shown to phosphorylate Akt at serine 473, it is thought that phosphorylation of Akt at this residue

can be effected by other kinases present in the cell, depending on the conditions under which Akt is activated. Regulation of Akt is further dependent on the levels and activity of phosphatases like PTEN(105,165), SHIP(98), PHLPP(106,107), PP2A (108)and proteins like CTMP(110).

MDM2 has no known kinase activity, though it has a defined ATP binding site. Most of its tumorigenic properties are attributed to the interaction of MDM2 with other proteins. The domains of MDM2 with which the different protein interact have been mapped out by previous studies.(54,166) Therefore information on what proteins could interact could be had using deletion mutants. This approach could give a broad idea on what mechanism may be employed by MDM2 in the activation of the Akt pathway.

b. Experimental Results:

A PI-3 kinase inhibitor Wortmannin inhibits MDM2-mediated Akt phosphorylation.

To determine if MDM2 requires the PI-3 kinase pathway to activate Akt phosphorylation, MDM2-mediated Akt phosphorylation was analyzed in the presence of Wortmannin, which can inhibit PI-3 kinase activity(167,168). H1299 cells stably expressing MDM2, or stably transfected with vector plasmid were treated with Wortmannin for 4 hours. Extracts from Wortmannin or vehicle (DMSO) treated cells were subjected to Western blot analysis as described earlier. The results of this analysis show that Wortmannin inhibits Akt phosphorylation as expected, and cells stably expressing MDM2 cannot activate Akt phosphorylation after treatment with

Wortmannin (Figure12B). This observation suggests that MDM2 up regulates PI3-kinase mediated Akt Phosphorylation.

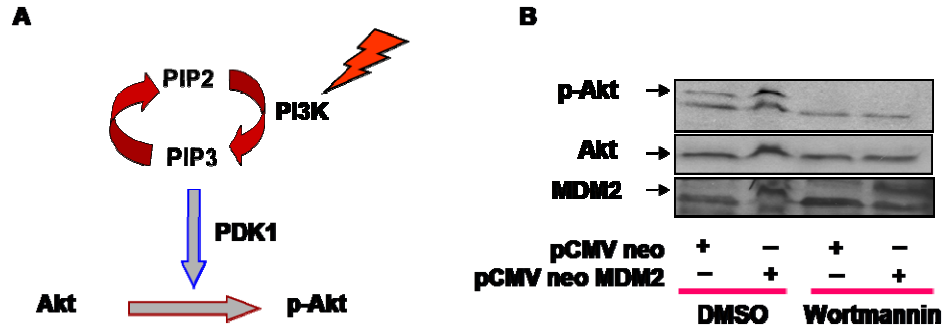


Figure12. MDM2 enhances Akt phosphorylation at Ser 473 through PI3-kinase pathway. Treatment of H1299 stable transfectants with Wortmannin, an inhibitor of PI3-kinase, inhibits MDM2-mediated Akt phosphorylation. p-Akt was identified with an antibody specific for Akt phosphorylated at Ser 473 (#9271 rabbit polyclonal, Cell Signaling); Akt (sc8312 rabbit polyclonal, Santa Cruz), MDM2 (AB1, Oncogene Science) and actin antibodies (AC-15, Sigma) were used to identify Akt, MDM2 and actin. Migration of the control MDM2, p-Akt and Akt bands are shown by arrows. The link between PI3-kinase and Akt phosphorylation is shown in a cartoon (A)

MDM2 enhances IGF-1mediated phosphorylation of Akt: IGF-1 has been shown by various groups to mediate Akt phosphorylation through the PI3K pathway(169-172). To further validate the role of the PI3K pathway in the activation of Akt due to MDM2 over-expression, we treated H1299 cells with IGF-1 for 10 minutes after nucleofecting with the MDM2 expressing plasmid. These cells were serum starved for 5 hours prior to the addition of the IGF-1. Our results (13A) showed that there was enhanced Akt phosphorylation in the presence of MDM2 when IGF-1 was added. Next, we silenced MDM2 in SJSA-1 cells and tested the response of the cells to IGF-1 treatment. We observed that silencing MDM2 reduced the phosphorylation of Akt by IGF-1 treatment

(13B). Together, these results indicate that MDM2 enhances the IGF-1 signaling. Since IGF-1 signaling works through the PI3K pathway it would seem that MDM2 serves to activate one or more components of the PI3K pathway.

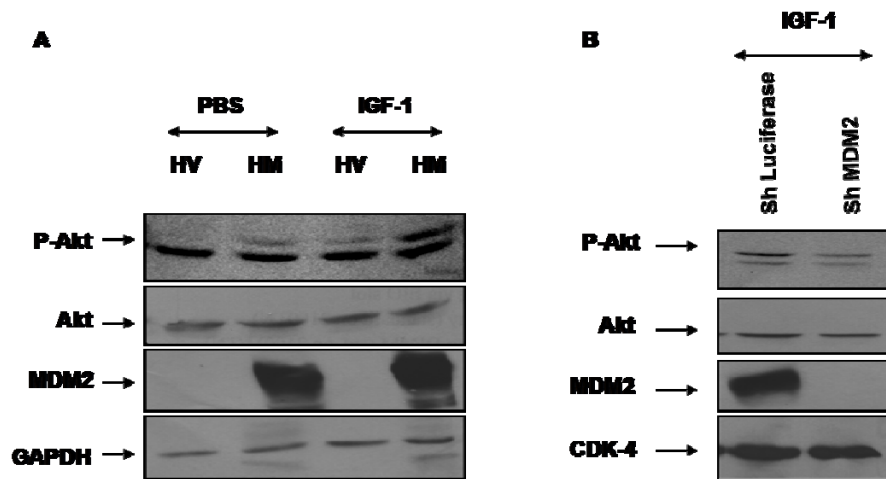


Figure13. MDM2 enhances IGF-1mediated phosphorylation of Akt. (A) Cell extracts of H1299 cells, serum starved 3 hours post nucleofection withMDM2 expression plasmids or empty plasmid and and treated with 50ng/ml IGF-1 for 10 minutes were analyzed by western blotting, 8 hours post nucleofection. Cell extracts of SJSA-1 cells (B) infected using lentivirus packaged with plasmids expressing shRNA against MDM2or non specific Luciferase for 4 days and treated with 50ng/ml IGF-1 for 10 minutes were analyzed by western blotting. p-Akt was identified with an antibody specific for Akt phosphorylated at Ser 473 (#9271 rabbit polyclonal, Cell Signaling); Akt (sc8312 rabbit polyclonal, Santa Cruz), MDM2 (AB1, Oncogene Science), GAPDH(sc32233,SantaCruz) and CDK4 (AB2,Oncogene Science) were used to identify Akt, MDM2,GAPDH and CDK4. Migration of the control MDM2, p-Akt and Akt bands are shown by arrows.

MDM2 does not influence phosphorylation of Akt at Threonine 308 residue: Akt activation requires the phosphorylation of Akt at threonine 308 and serine 473 residues. There is still a debate on whether threonine 308 phosphorylation follows phosphorylation

of Akt at serine 473 or occurs before it(99,164,173) Akt phosphorylation at threonine 308 residue is shown to require the PI3K pathway and the kinase PDK1.(100,101,163,164,173) Since MDM2 requires the PI3K pathway for the activation of Akt at serine 473 residue, we checked whether MDM2 over-expression leads to increase in Threonine 308 phosphorylation of Akt. This would also indicate that both events happen when Akt is docked to the membrane and that the increased Akt phosphorylation happens by the induction of some upstream signaling pathway by MDM2. Our data shows that there is no increase in phosphorylation of Akt at threonine 308 due to the over-expression of MDM2 (Figure 14B). This could mean that either MDM2 mediates phosphorylation of Akt specifically at serine 473 or that the threonine 308 activation pathway in H1299 is deregulated.



Figure14. MDM2 does not influence phosphorylation of Akt at threonine 308 residue. Cell extracts of H1299 stable transfectants were analyzed by western blotting. p-Akt was identified with an antibody specific for Akt phosphorylated at Threonine 308 (#9275rabbit polyclonal, Cell Signaling) and actin antibodies (AC-15, Sigma) was used to actin. Migration of the p-Akt and actin bands are shown by arrows. The cartoon (A) shows the 2 phosphorylation residues of Akt required for its full activation.

MDM2 does not influence the cross talk of ERK pathway and Akt: Similar to the Akt activation of MDM2, the Ras –Raf- MEK-ERK pathway has also been shown to lead to phosphorylation of MDM2 and subsequent degradation of p53(174). Also there exists a crosstalk between the Ras –Raf- MEK-ERK and the PI3K-Akt pathway on the level of Raf-1 and Akt. Depending on the cell type and conditions, the two pathways have been shown to either act synergistically for survival or antagonistically with the Erk activation leading to growth arrest and Akt pathway leading to proliferation(175). Therefore we determined if there existed any correlation with MDM2 expression and the activation of the Ras-Raf- MEK-ERK pathway. If such a correlation existed, it could serve as a lead for a possible mechanism of activation of the Akt pathway. We checked initially if over-expression of MDM2 could modulate the phosphorylated MAP kinases (ERK1 and ERK2). We chose both diploid fibroblast where we over-expressed MDM2 and the MDM2 over-expressing stable H1299 cells and determined the levels of p-ERK1/2. Our results (Figure 15) show that MDM2 does not activate or lower the p-ERK1/2 levels. Therefore our observation was specific to the PI3K- Akt pathway.

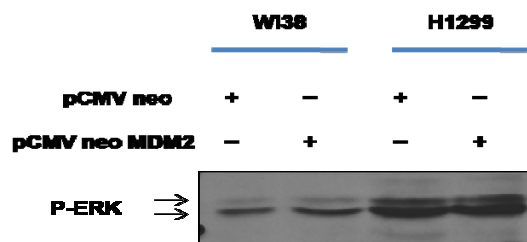


Figure15. MDM2 does not influence the cross talk of ERK pathway and Akt. Cell extracts of H1299 stable transfectants and WI38 trasfected with 4µg of MDM2 or corresponding vector expressing plasmid, were analyzed by western blotting. p-ERK1/2 was identified with an antibody specific for pERK(V-8031) from Promega

Silencing Rictor does not inhibit the ability of MDM2 to activate Akt at serine 473.

The mammalian target of Rapamycin/ Rapamycin Insensitive Companion of mTOR (mTOR/Rictor) complex is a kinase known to be able to phosphorylate Akt at serine473(99). Since we did not observe any change in the levels of threonine 308 phosphorylation of Akt when MDM2 was over-expressed, we determined whether this serine 473 specific kinase complex played a role in our observation. For this purpose, we made lenti virus packaged with a plasmid expressing Rictor shRNA and infected the H1299 cells for 4 days, and then nucleofected with MDM2 expressing plasmid or control empty vector. Our results show that silencing Rictor in the MDM2 over-expressing H1299 cells did not reduce the levels of Akt phosphorylated at serine 473 and that it was enhanced in comparison with the H1299 transfected with a plasmid expressing just the vector (Figure 16A). This verified that MDM2 does not require the mTOR/ Rictor complex to induce phosphorylation of Akt at serine 473 residue. Taken together with the fact that the PI3K pathway is required for the activation, it would seem to suggest that the pathway leading to the phosphorylation of threonine 308 residue is deregulated.

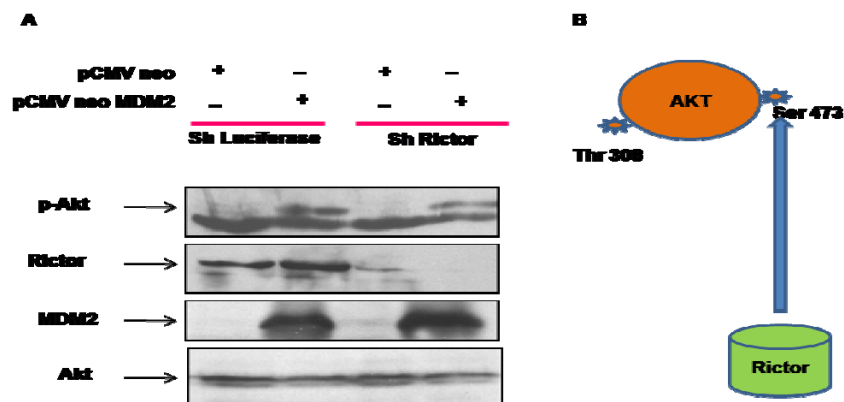


Figure16. Silencing Rictor does not inhibit the ability of MDM2 to activate Akt at serine 473. Cell extracts of H1299 infected using lentivirus packaged with plasmids expressing shRNA against Rictor or non specific luciferase for four days and then nucleofected with either empty vector or vector expressing MDM2 were prepared and analyzed by western blotting. p-Akt was identified with an antibody specific for Akt phosphorylated at Ser 473 (#9271, Cell Signaling); Akt (sc8312 rabbit polyclonal, Santa Cruz), MDM2 (AB1, Oncogene Science) and Rictor (#2114, Cell signaling), antibodies were used to identify Akt, MDM2 and Rictor. Migration of the control MDM2, Rictor, p-Akt and Akt bands are shown by arrows. The link between Rictor and Akt phosphorylation is shown in a cartoon (B)

MDM2 over-expressing cells do not show decreased levels of PHLPP and CTMP

transcripts. Regulation of the PI3K/Akt pathway is also controlled by cellular phosphatases. Since H1299 has suppressed PTEN expression due to promotor methylation and our observation was specific to the serine 473 residue, we decided to look at the the pH domain and leucine rich repeat protein phosphatase (PHLPP), which was specific for dephosphorylation of the serine 473 residue(106,107). We also looked at the Carboxy Terminal Modulating Protein (CTMP) which binds to Akt and dephosphorylates it(109). We checked for the transcript levels of the phosphatases in the cell to find out if MDM2 over-expression led to their reduced levels. Our data shows that there is no significant change in the transcript levels of both PHLPP (Figure 17A) and CTMP (Figure17b) in the presence of MDM2.

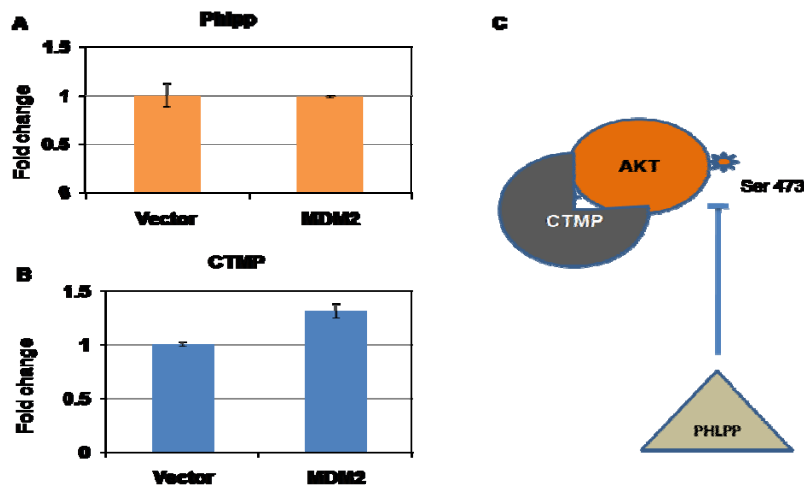


Figure17. MDM2 over-expressing cells do not show decrease levels of PHLPP and CTMP transcript levels. Normalized fold change in transcript levels determined by qPCR are shown by bar graphs. An endogenous GAPDH control was used to ensure equal mRNA levels in each sample. The normalized transcript levels of (A) PHLPP and (B) CTMP are shown. Experiments were done as technical triplicates and error bars indicating the standard deviations is shown (C) Cartoon depicting the dephosphorylation events of Akt by CTMP and PHLPP.

MDM2 over-expressing cells show enhanced CXCL8 transcript levels and faster

migration rate: Interleukin 8(IL8, CXCL8) is a cytokine that has been shown to increase cell proliferation in non small lung cancer cells similar to our observation with MDM2 over-expression(176) (Figure 10). Interestingly, IL8 has been shown to activate the Akt pathway through the PI3K pathway, after phosphorylation of Cbl.(177)

Therefore we checked the levels of CXCL8 in our MDM2 over-expressing stable H1299 cell lines. Our results show that MDM2 over-expressing stable cells expressed enhanced transcript levels of IL-8 (Figure18A). Since IL-8 has been implicated in promotion of migration(178), we did a migration/scratch assay in our cells and found that MDM2 over-expressing cells migrate faster than control cells (Figure18B). Therefore by

increasing CXCL8 levels, MDM2 could activate the Akt pathway and this could be a mechanism by which we see increased activation of Akt in the presence of MDM2.

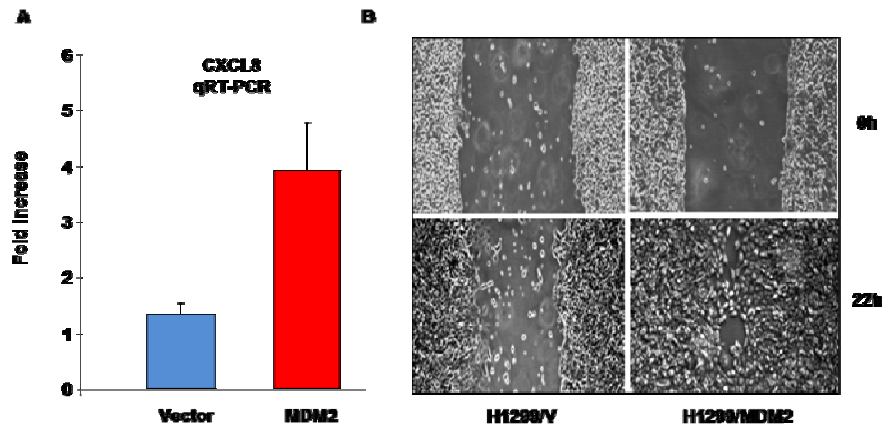


Figure18. MDM2 over-expressing H1299 cells show enhanced CXCL8 transcript levels and faster migration rate. (A) Normalized fold change in transcript levels of CXCL8 determined by qPCR is shown by bar graphs. An endogenous Actin control was used to ensure equal mRNA levels in each sample. Triplicates of the samples were analyzed and error bars representing standard deviations are shown. (B) Migration assay was performed on H1299 stable transfectants by scratching the plate with a pipette tip and allowing the cells to grow. The scratch closure was analyzed after 24 hours.

The p53 interaction domain of MDM2 is dispensable for Akt phosphorylation.

Since MDM2 is a multifunctional protein, and many of its functional domains have been identified, we determined the domain of MDM2 responsible for activating Akt phosphorylation. For this purpose MDM2 or its C- or N-terminal deletion mutants were transiently over-expressed in H1299 cells as described earlier(179). To determine the consequence of stable expression, H1299 cell lines stably expressing C- and N-terminal deletion mutants of MDM2 were also generated. Extracts prepared from these cells were subjected to Western blot analysis to detect Akt phosphorylation and expression of

MDM2 deletion mutants. Our data (Figure 19 B,C) show that an N-terminal deletion mutant of MDM2 (Del 1-120) lacking 120 amino acid residues is capable of activating Akt phosphorylation, whereas a C-terminal deletion mutant of MDM2 (Del 491-155) harboring N-terminal 154 amino acids is inactive in Akt phosphorylation. Thus the Akt phosphorylating activity of MDM2 is located within 121 to 491 amino acid residues which is C terminal to the p53 binding domain although the sequences at the N-terminal of MDM2 may be necessary for optimal activity.

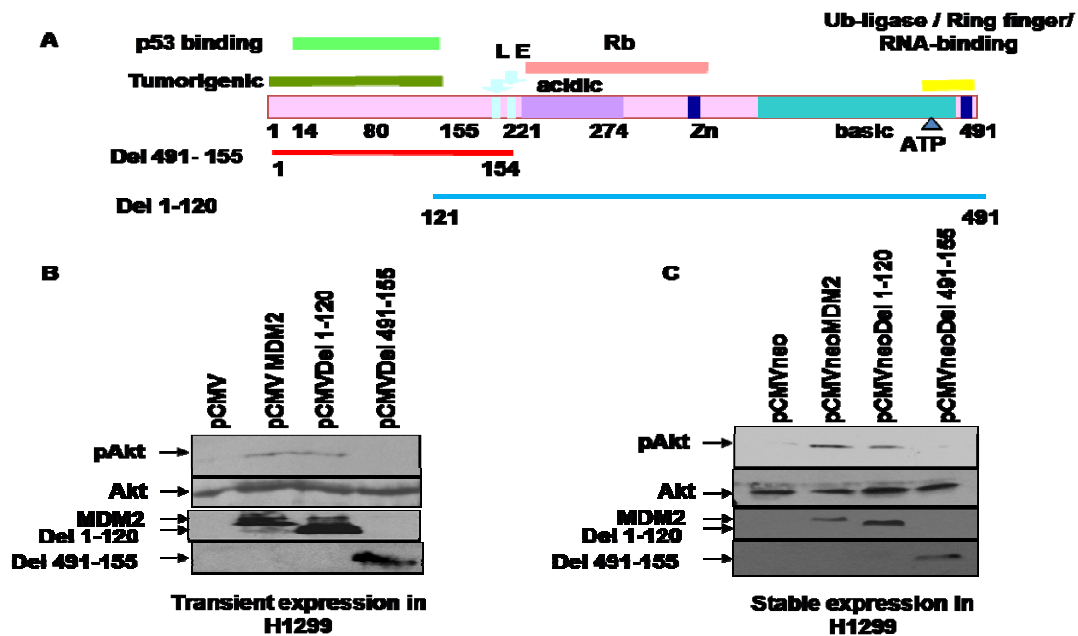


Figure19. The p53 interaction domain of MDM2 is dispensable for Akt phosphorylation. The known functional domains of MDM2 are indicated (A). Akt phosphorylating ability of the full-length and deletion mutants of MDM2 was determined by Western blot analysis after their transient (B) and stable (C) expression in H1299 cells. A mouse monoclonal antibody 2A10 was used to detect the N-terminal deletion mutant of MDM2 (Del 1-120), and a rabbit polyclonal antibody N20 was used to detect the C-terminal deletion mutant Del 491-155. Other antibodies have been described in Figure 4.

The Akt phosphorylating domain of MDM2 is responsible for altering Etoposide sensitivity. Since our data show that Akt phosphorylating activity of MDM2 is required for MDM2-mediated alteration of Etoposide sensitivity (Figure 9), we tested whether N-terminal 120 amino acid residues of MDM2 is required for this function. For this purpose equal number of H1299 stable transfectants expressing full-length MDM2 or its deletion mutants (Del 1-120 and Del 491-154) were plated, and their sensitivity to Etoposide was determined by colony development. The results (Figure 20) show that H1299 cells stably expressing MDM2 generated higher number of colonies than H1299 cells stably transfected with the vector. The stable transfectants expressing the C-terminal deletion mutant Del 491-155 did not show this effect, whereas the N-terminal deletion mutant Del 1-120 also generated higher number of colonies than vector stable transfectants. These data suggest that the Akt phosphorylating activity of MDM2 is co-located with its ability to enhance Etoposide tolerance of the cancer cells.

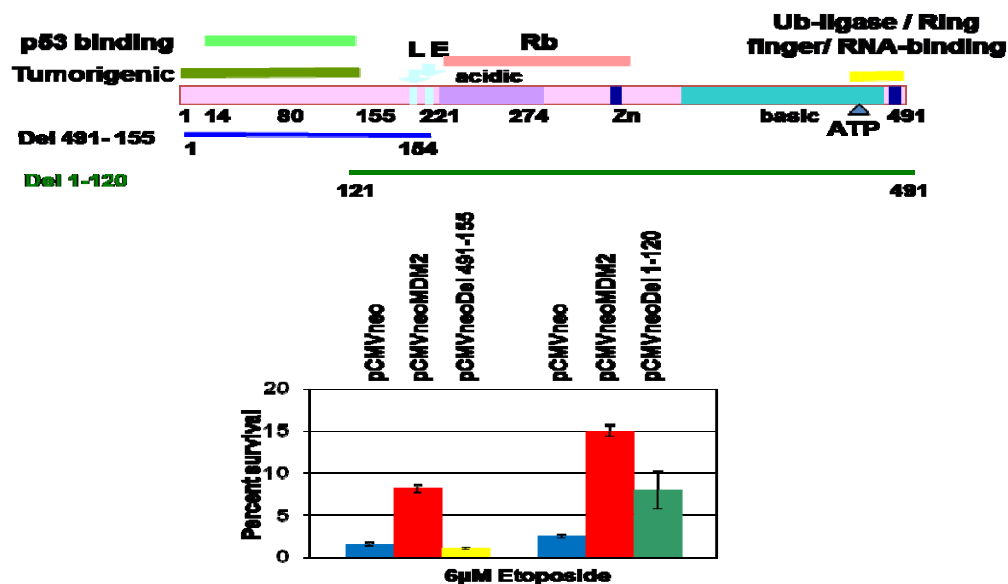


Figure20. The Akt phosphorylating domain of MDM2 is responsible for altering Etoposide sensitivity. Bar graph to compare number of colonies generated after treatment of H1299 stable transfectants expressing full length, either deletion mutants or control vector with 6 μ M Etoposide or DMSO. The results shown are representative of 2 different experiments, with the vector and full length MDM2 harboring stable cells common to both. Percent survival was calculated considering number of colonies generated in the vehicle treated plates to be 100%. The experiments differed in the use of the deletion mutant. Experiment was done in triplicates and error bars representing standard deviations are shown.

Inhibition of Akt phosphorylation renders the cells expressing deletion mutant Del 1-120 sensitive to Etoposide treatment. To show that Del1-120 expressing cells provided resistance to Etoposide by augmenting the Akt pathway, we used the Akt inhibitor, SH-6 to inhibit the Akt phosphorylation and performed clonogenic assays as described earlier with Etoposide. Our results clearly shows that Sh-6 inhibits Akt phosphorylation(Figure 21B) and these cells became sensitive to the treatment with Etoposide (Figure21A).

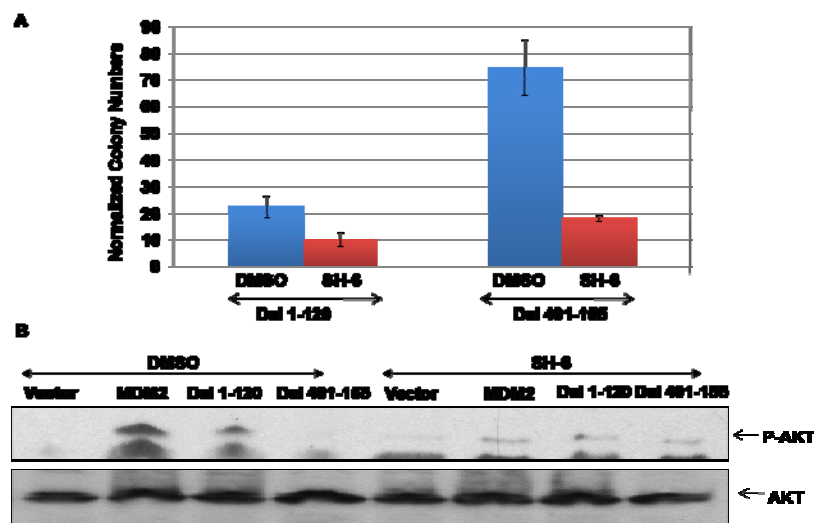


Figure21. Inhibition of Akt phosphorylation renders the cells expressing deletion mutant Del 1-120 sensitive to Etoposide treatment. Panel (A) shows a bar graph to compare number of colonies generated after treatment of H1299 stable transfectants expressing deletion mutants with 1.76 μ M SH-6 or DMSO and colony formation assay with 6 μ M Etoposide. Panel (B) shows inhibition of Akt phosphorylation by SH-6. Total Akt is used as loading control and indicated.

The phosphorylated Akt-MDM2 interaction domain is required for the increased Akt phosphorylation in the presence of MDM2. To fine map the domain of MDM2, we performed further deletion mutant analysis with deletion mutants 1-189 and deletion mutants 491-220(179) H1299 cells were nucleofected with 4μg of plasmids expressing either full length, deletion 1-189, deletion 491-220 or control vector, harvested after 8 hours and analyzed by western blotting. Our results show that both the deletion mutants and full length MDM2 enhance the levels of Akt phosphorylation (Figure 22B). Taken together with our previous deletion mutant analysis, we inferred that the region between amino acids 189-220 was required for our observation. Interestingly, work from other laboratories have shown this to be the region that interacts with phosphorylated Akt in the cytoplasm which leads to phosphorylation of MDM2 and the consequent entry of MDM2 into the nucleus.

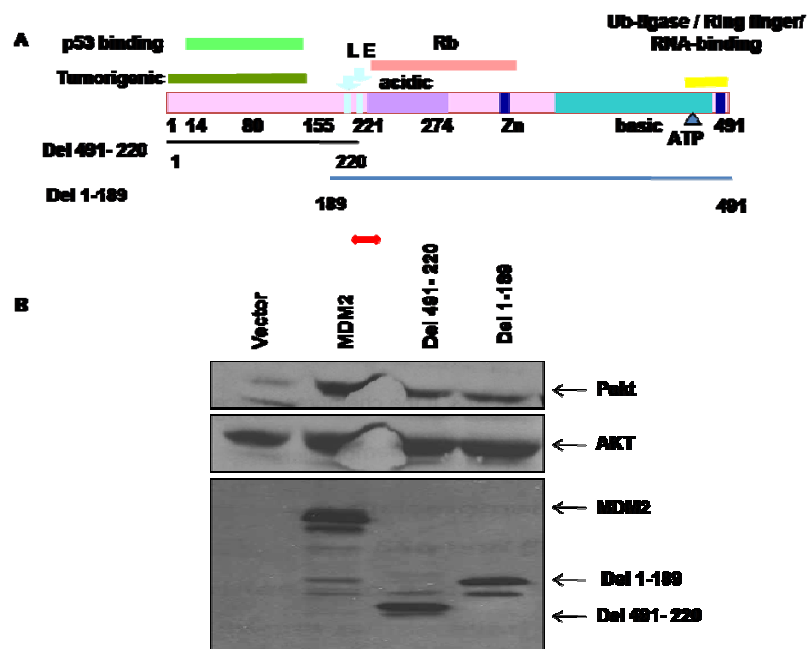


Figure22. The phosphorylated Akt-MDM2 interaction domain is required for the increased Akt phosphorylation in the presence of MDM2. The known functional domains of MDM2 are indicated (A). Akt phosphorylating ability of the full-length and deletion mutants of MDM2 were determined by Western blot analysis after their transient expression in H1299 cell line (B). A mouse monoclonal antibody 2A10 was used to detect the N-terminal deletion mutant of MDM2 (Del 1-89), and a rabbit polyclonal antibody N20 was used to detect the C-terminal deletion mutant Del 491-220. Other antibodies have been described in Figure 4.

c. Chapter Summary.

To determine the mechanism by which MDM2 activates the Akt pathway, we tested the importance of the PI3K signaling pathway and found that it was required for the activation. As shown by the addition of IGF-1, there is an augmentation of Akt phosphorylation which seems to be an upstream signaling event. However, there is no change in the level of the Threonine 308 phosphorylation on Akt. It could very well be that the pathway leading to the phosphorylation of Threonine 308 could be deregulated and this requires further investigation. To further investigate the point that Akt may require the PI3K pathway for its activation in the presence of MDM2, we show that Rictor has no role in this MDM2 mediated Akt phosphorylation. Also transcript levels of known phosphatases like PHLPP and Akt binding protein CTMP which serves to de phosphorylate Akt are not shown to be reduced. Since these seem to suggest an extracellular membrane bound receptor mediated signaling event, we checked if there is any increase in IL-8 transcript levels and find that it is higher in the presence of MDM2. Also supporting the result is the fact that MDM2 over-expressing cells migrate faster. So this seems to be a plausible mechanism by which MDM2 activates Akt; more experiments are needed to confirm it.

Since the domains of MDM2 are well mapped out and their interacting partners have been studied over the years, we did a deletion analysis to figure out what domain(s) of MDM2 was/were responsible for our observation. Interestingly, we found that the region of MDM2 implicated by others to be responsible for binding to Akt in the cytoplasm is the region, which when present augments Akt phosphorylation in our experiments. This falls well into our hypothesis that MDM2 would need Akt phosphorylation for its oncogenic properties like increased growth, migration and survival.

L. Chapter 5

Determination of events downstream of MDM2 mediated Akt phosphorylation.

The manuscript for the work presented in this chapter is currently under preparation.

a. Introduction. The PI3K/Akt pathway activation has been shown to be involved in proliferation, migration, survival, insulin signal transduction and chemotherapeutic resistance through the phosphorylation of downstream substrates(89,90) (Figure3). Akt activation mediates many of the cellular effects of insulin. Insulin mediated Akt activation has been shown to be severely diminished in patients with Type 2 diabetes. The targets in this pathway include the Glycogen Synthase Kinase (GSK3)(180,181), Mammalian Target of Rapamycin (mTOR) (182,183)and Insulin Receptor Substrate(IRS-1)(184,185) among others. Over-expression of Akt has been shown to have an anti apoptotic effect in many cell types. The components of the apoptotic machinery including the Bcl-2 family proteins, caspases and transcriptional regulators of apoptosis like Fas ligands, have been shown to be influenced by the Akt activation(89,113,114,118). Akt has been shown to increase proliferation and migration by its ability to phosphorylate the I κ B kinases (IKKs) and thereby activate the NF κ B pathway(121-123). Akt activation has also been shown to increase in cyclin D levels(124,125). PKB has also been shown to be involved in inducing angiogenesis by activating endothelial Nitric Oxide Synthase (eNOS), resulting in the sustained production of Nitric Oxide by endothelial cells(186-188).

We therefore set out to determine if MDM2 mediated Akt phosphorylation can result in the activation of known substrates of Akt and to try and map out the pathways that result in MDM2 mediated increased proliferation, migration and chemotherapeutic resistance.

b. Experimental results.

MDM2 over-expressing cells show increased GSK3 α/β phosphorylation: A well known target of Akt is the kinase GSK3, a component of the Wnt pathway. GSK3 α and GSK3 β have shown to be involved in the phosphorylation and degradation of β catenin, thus inhibiting β catenin's transcriptional effects. The activity of GSK3 is normally suppressed in proliferating cells by phosphorylation mediated by Akt and other kinases(126-128). We therefore looked at whether MDM2 over-expression led to the modulation in the levels of GSK3 phosphorylation. We observed that while MDM2 expressing cells had enhanced levels of phosphorylated GSK3 α/β (Figure 23A), treatment of the cells with Sh-6 abrogates it (Figure23B). Further, silencing of endogenous MDM2 in SJSA-1 cells lowers the phosphorylation of GSK3 α/β . Therefore these results seem to suggest that MDM2 mediated Akt phosphorylation results in the phosphorylation of downstream GSK3 α/β .

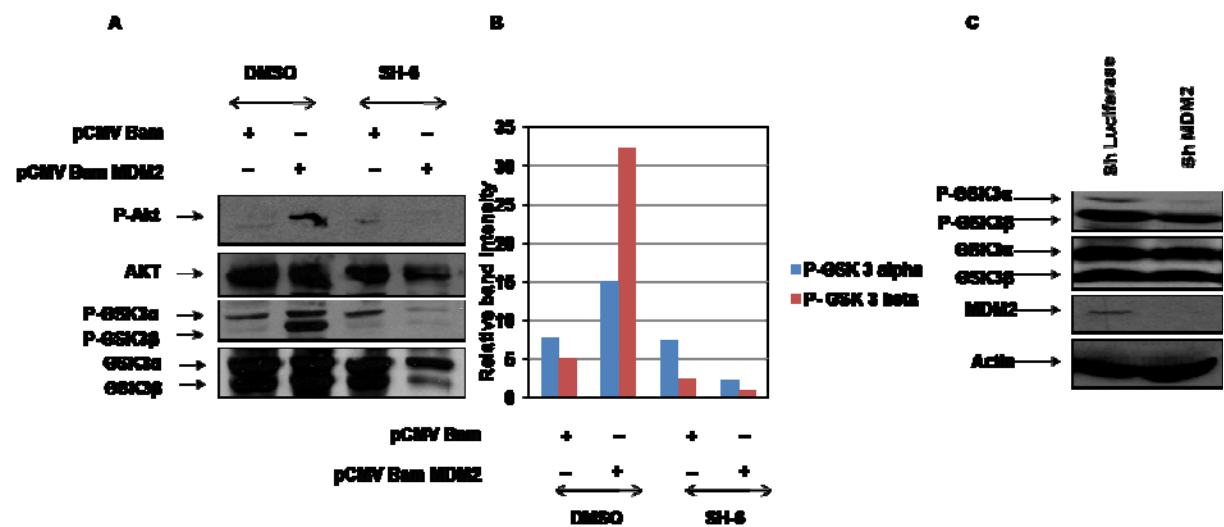


Figure23. MDM2 over-expressing cells show increased GSK3 α / β phosphorylation: (A) H1299 stable transfectants expressing MDM2 or empty vector were pretreated with 1.76 μ M SH-6 or DMSO for 4 hours, harvested and analyzed by western blotting.(B) Densitometric analysis on (A) performed on the P-GSK3 α and P-GSK3 β bands after normalizing the intensity with total GSK3 α and total GSK3 β . (C) SJSA-1 cells were infected with lentivirus packaged with plasmid expressing either shRNA against MDM2 or non specific luciferase as control for 4 days, harvested and analyzed by western blotting. Antibody for GSK3 α / β phosphorylated at ser 21/9 (#9331) was from Cell Signaling Technologies and GSK3 α / β (sc-56913) from SantaCruz Biotechnologies. Other antibodies are mentioned in Figure 4. Migration of the proteins are shown by arrows.

Induction of MDM2 elevates known Akt targets involved in proliferation, migration

and chemo resistance pathways: Since MDM2 over-expression led to enhanced Akt phosphorylation and increased proliferation, migration and resistance to chemotherapeutic agents, we decided to do a RT profiler PCR array (SuperArray) to get a broad idea of which genes are differentially regulated by MDM2 over-expression(189,190). Since the functions of most of the genes in the PI3K/Akt pathway have been well characterized, this would give us an insight on the various pathways that were getting activated because of MDM2 mediated Akt phosphorylation. We developed a Ponasterone inducible clone that expressed MDM2 when Ponasterone was added to the media (Figure24A)(191,192). We isolated the RNA, made cDNA and used it in our array consisting of 84 genes. The genes that showed the most significant changes are shown (Figure 24C). Genes like Fibronectin and PECAM-1 play a major role in cell adhesion, growth, migration and differentiation, and are important for processes such as wound healing and embryonic development. These have been shown to be able to activate the Akt pathway(193). Further study will confirm if they are involved in the mechanism by which MDM2 enhances Akt phosphorylation. The Activator Protein1/cJun transcription factor has been implicated in various cancers and has been shown to be activated by the Akt pathway(194). IL8 expression was enhanced just as

what we saw with our stable MDM2 expressing H1299. Genes like IKK beta, INOS, IL2 and IL8 have been shown to be important members of the NF- κ B pathway with IKK beta phosphorylation activating the pathway and INOS, IL2 and IL8 are targets downstream; all of which have been shown to be activated by Akt pathway signaling.(195-197). Ornithine Decarboxylase has been shown to be regulated by insulin signaling(198)`. The links between PKC alpha and PKC epsilon and the Akt pathway is well studied(199,200). The most significant of our result was the anti apoptotic gene Bcl2, which showed a 96 fold increase. BCL_{xL} was also higher, which seemed to imply that the anti-apoptotic pathway was activated when MDM2 is induced. Since Bcl2 levels have been shown to be up regulated when the Akt pathway is activated(113), this may be the pathway by which MDM2 increases Bcl-2 levels.

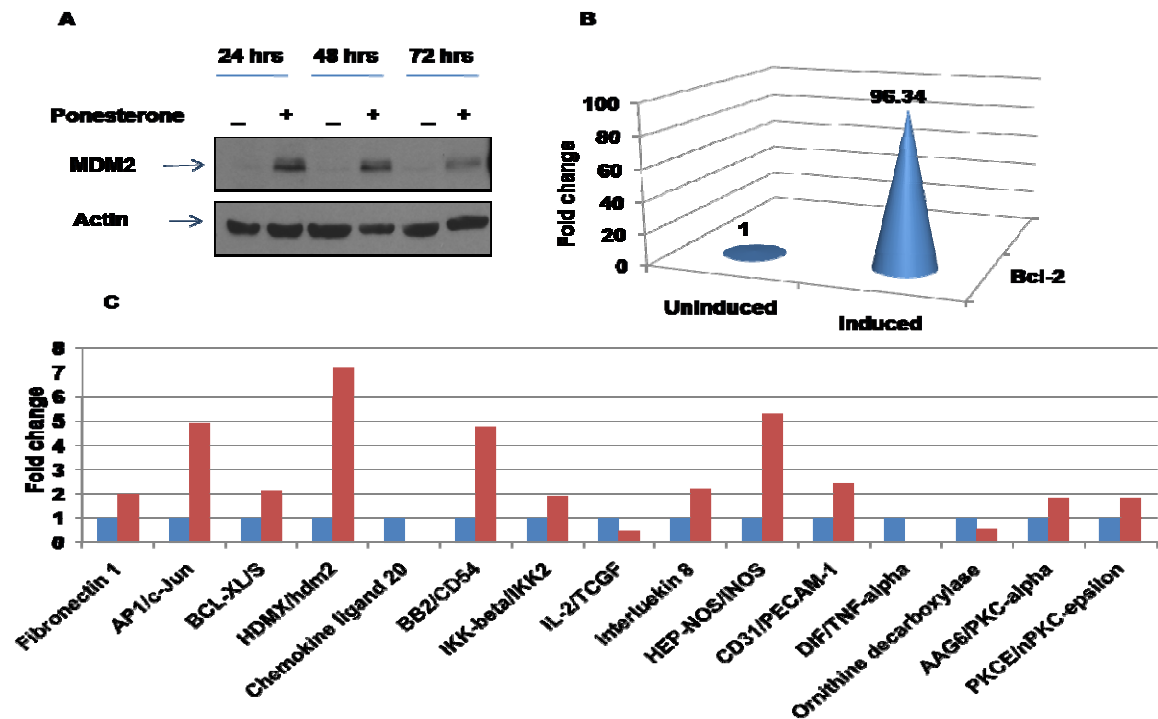


Figure 24. Induction of MDM2 elevates known Akt targets involved in proliferation, migration and chemo resistance pathways: (A) A ponasterone inducible clone was harvested after treatment with either 5 µg/ml final concentration ponasterone or equal volume of vehicle, harvested at 24, 48 and 72 hours after addition of ponasterone and analyzed by western blotting. Antibodies used are mentioned in Figure 4 and migration of the proteins shown by arrows. (B, C) Normalized fold change in transcript levels of the labeled genes determined by qPCR array shown by bar graphs. An endogenous GAPDH control was used to ensure equal mRNA levels in each sample.

MDM2 induces Bcl-2 expression both at transcriptional and protein levels: Since MDM2 offers resistance to chemotherapeutic agents and induces Akt phosphorylation, we looked at the proteins which are known Akt targets and have been implicated in drug resistance. Bcl-2 has been shown to be induced by Akt phosphorylation(113) and its increased levels have been correlated to resistance to Paclitaxel and Etoposide resistance(201-204). Our qPCR array result showed that there was a 96 fold increase of Bcl-2 transcripts when MDM2 was induced in H1299 cells. Therefore we decided to confirm if the same was true in our H1299 cells, stably expressing MDM2. Our results show that MDM2 induces Bcl-2 expression both transcriptionally (Figure 25A) and at the level of protein (Figure 25B). To further confirm whether endogenous MDM2 plays a role in the modulation of Bcl-2 levels, we analyzed SJSA-1 cells stably expressing shRNA against MDM2 (Figure 5B) for Bcl2 levels transcriptionally and found that the cells where MDM2 was silenced had the Bcl2 expression repressed (Figure 25C). Further we infected SJSA-1 cells with lentivirus packaged with plasmids expressing shRNA against MDM2 or non specific luciferase for control and harvested the cells after 4 and 5 days for western analysis. As shown (Figure 25D), silencing of MDM2 decreases the protein levels of Bcl-2.

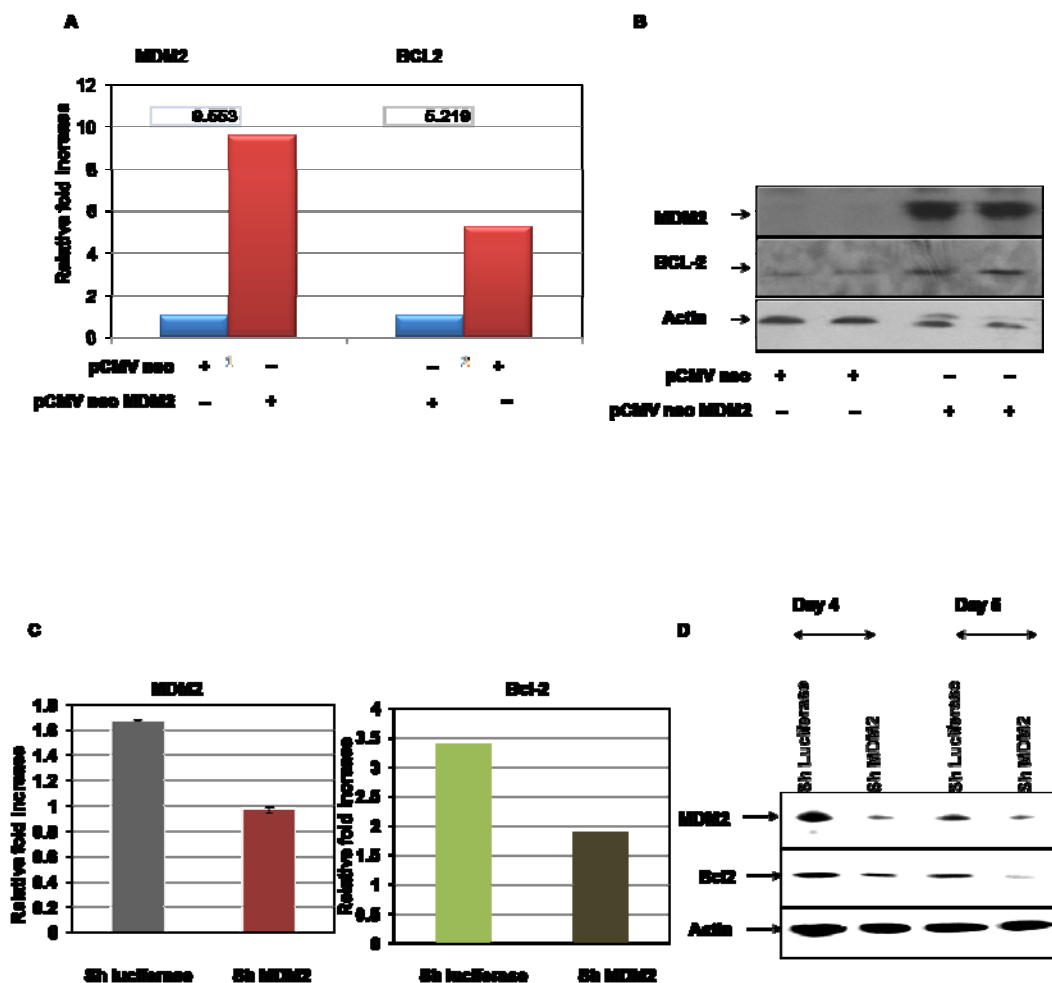


Figure25. MDM2 induces Bcl-2 expression both at transcriptional and protein levels. Normalized fold change in transcript levels of MDM2 and Bcl-2 determined by qPCR is shown by bar graphs (A, C). An endogenous GAPDH control was used to ensure equal mRNA levels in each sample. Experiments were conducted as technical triplicates with error bars shown indicating standard deviations (B). Experiments have been repeated and show similar trend. H1299 stable transfectants expressing MDM2 and control cells were harvested and analyzed by western blotting (D). SJSA-1 cells were infected with lentivirus packaged with plasmids expressing either shRNA against MDM2 or non specific luciferase for either 4 or 5 days, harvested and analyzed by western blotting. The antibody for Bcl-2 (610538) was from BD Biosciences. Other Antibodies are as in Figure 4.

MDM2 induces enhanced processing and increased nuclear localization of NFκB2.

Gustin et al showed that activation of the Akt pathways induced the processing of the non canonical NFκB pathway precursor p100 to p52(205). The processing has been implicated in the up-regulation of Bcl-2 and also in cellular processes such as proliferation, migration and resistance to chemotherapeutic agents(206-208). Therefore we studied if MDM2 expressing cells show higher level of NFκB2 processing. Our result (Figure 26A) shows that when MDM2 is induced in H1299, it leads to enhanced processing of NFκB2. Since NFκB2 is a transcription factor(206-208), we decided to check if we have more processed form of the protein in the nucleus when MDM2 is over-expressed. We therefore did a cytoplasmic and nuclear extraction of H1299 stable transfectants and looked for the levels of processed and unprocessed form of NFκB2. We see (Figure 26B) that there is more nuclear localization of NFκB2 when MDM2 is stably expressed.

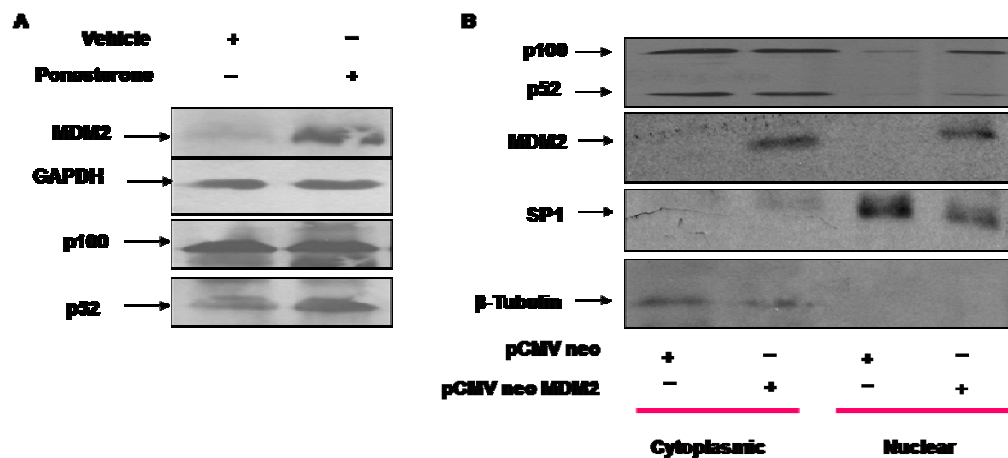


Figure 26. MDM2 induces enhanced processing and increased nuclear localization of NFκB2. (A) The ponasterone inducible clone was treated with 5μg/ml ponasterone or equal volume of vehicle for 24 hours, harvested and analyzed by western blotting. (B) Cytoplasmic and nuclear extracts of the stable H1299 transfectants expressing MDM2 or empty vector were made and analyzed by western blotting. β Tubulin is used as cytoplasmic marker and Sp1 as nuclear marker. Antibodies for NFκB2 (#05-361) was from Upstate Biotechnologies and Sp1 (sc-59 1) from Santa Cruz Biotechnologies.

Silencing of NFκB2 sensitizes the MDM2 over-expressing cells to Paclitaxel, but not Etoposide treatment. Since NFκB2 has been implicated in the resistance to chemotherapeutic agents(206-208), we tested if our MDM2 over-expressing cells offered resistance to Etoposide and Paclitaxel treatment through the NFκB2 pathway. Therefore we infected H1299 stable transfectants with lentivirus packaged with plasmids expressing shRNA against NFκB2 and silenced the protein levels of NFκB2 (Figure 27A). These cells were then plated in equal numbers and clonogenic assays were performed to test their resistance to Etoposide or Paclitaxel. We observed that there is no effect in the resistance to Etoposide when the NFκB2 in the MDM2 over-expressing H1299 is silenced (Figure 27B). However silencing NFκB2 sensitizes the cells to Paclitaxel; the levels of NFκB2 in the cell correspond to the survival levels after drug treatment (Figure 27C). Therefore it can be concluded that MDM2 offers resistance to Paclitaxel through the NFκB2 pathway.

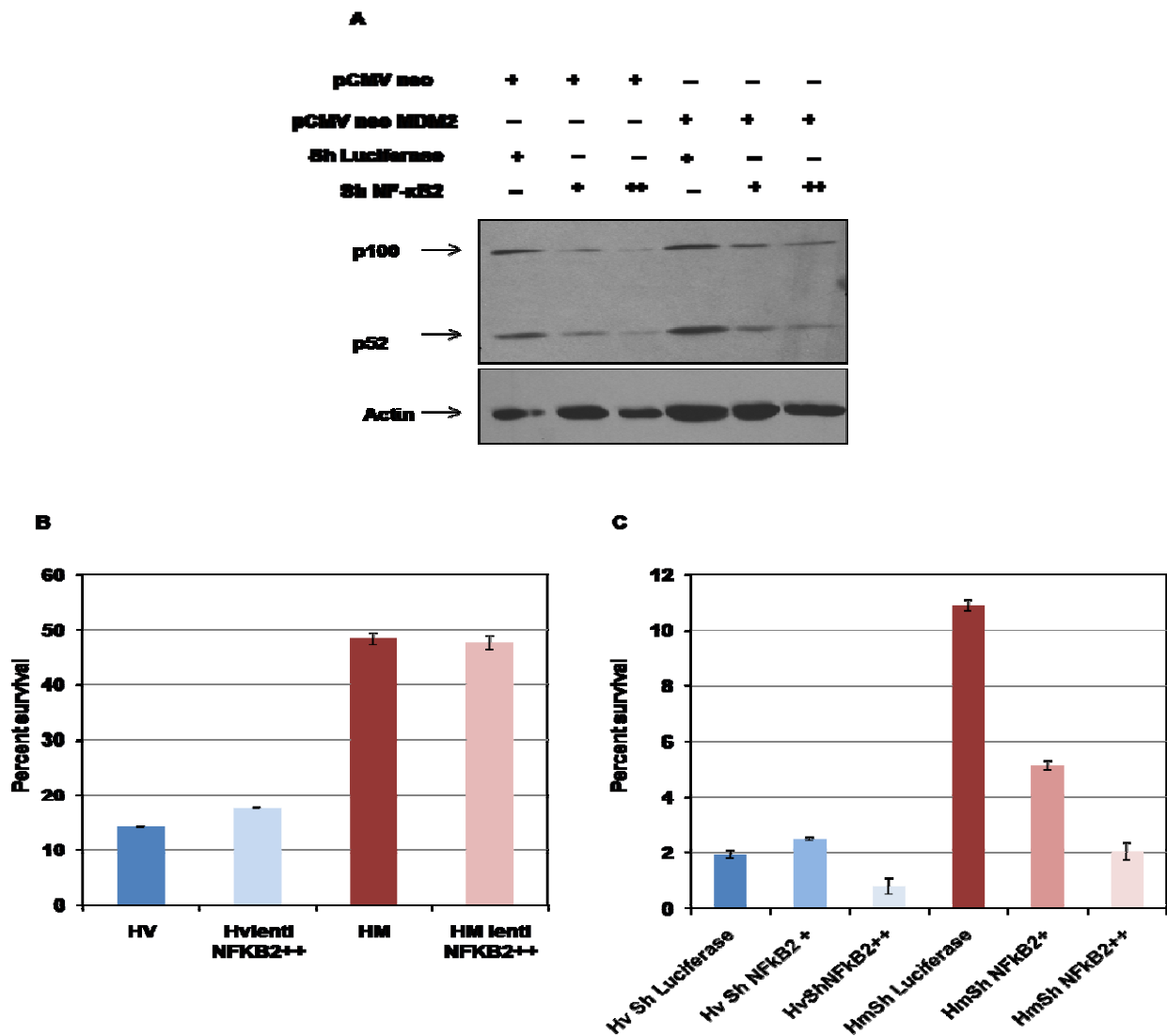


Figure 27. Silencing of NF κ B2 sensitizes the MDM2 over-expressing cells to Paclitaxel, but not Etoposide treatment (A) Stable transfectants of H1299 expressing either MDM2 or empty vector were infected with lentivirus packaged with plasmids expressing either shRNA against NF κ B2 or non specific luciferase. Arrows indicate migration of respective proteins. Clonogenic assays performed on NF κ B2 silenced cells using (B) 1 μ M Etoposide or (C) 25nM Paclitaxel. Percent survival was calculated considering number of colonies generated in the vehicle treated plates to be 100%. Experiments done in triplicates and error bars indicating standard deviations are shown.

Silencing NFκB2 decreases proliferation rate. The NFκB2 pathway has been correlated with enhancement of proliferation rates in cells (209). Since we observed increased growth rate and more processing of NFκB2 when MDM2 is over-expressed, we decided to test the role of NFκB2 in proliferation. We silenced NFκB2 in H1299 cells using siRNA (Figure 28A) and plated equal number of cells for a growth assay. The result (Figure 28B) clearly shows that lowering of NFκB2 reduces the growth rate of the cells.

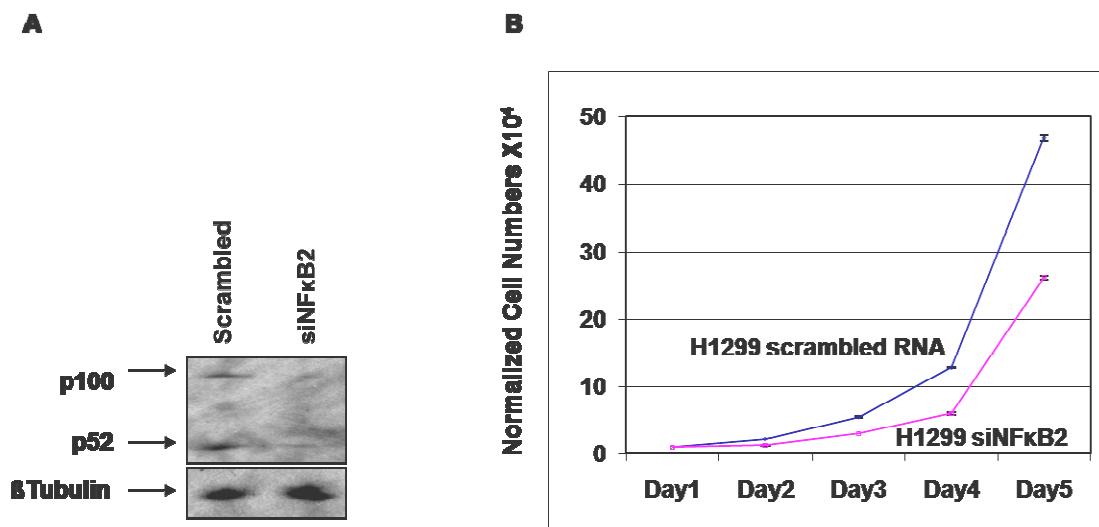


Figure28. Silencing NFκB2 decreases proliferation rate. (A) H1299 cells nucleofected with 80 pm siRNA against NFκB2 or non specific scrambled control was harvested 24 hours post nucleofection and analyzed by western blotting. (B) Equal number of scrambled RNA or siNFκB2 treated H1299 cells were plated 24 hours after nucleofection. The plates were harvested every 24 hour post plating and counted using a coulter counter. The experiment was conducted in triplicates and error bars are representing standard deviations of the cell counts are shown.

c. Chapter Summary. To identify the consequence of MDM2 mediated Akt phosphorylation, we looked at the players involved in the cell processes mediated by Akt- including but not limited to insulin metabolism, proliferation, migration and survival. One of the most studied downstream event of Akt phosphorylation is the phosphorylation of GSK3. We see enhanced GSK3 phosphorylation in the presence of MDM2 and we show that it is mediated by Akt phosphorylation. Also silencing MDM2 reduces the phosphorylated GSK3 levels. A broad idea of the consequence of MDM2 over-expression is demonstrated by the qPCR array we performed using cDNA prepared from an inducible MDM2 system. The resulting modulation of genes, which have been shown to be involved in the Akt pathway, reinforces our observation of MDM2 mediating the expression of the Akt pathway. Further we show that stable MDM2 over-expressing cells have increased transcription and protein levels of anti apoptotic Bcl2. Also silencing endogenous MDM2 leads to reduction in Bcl2 levels. Bcl2 levels have been correlated with Akt phosphorylation and resistance to drugs like Etoposide and Paclitaxel. This could be a mechanism of drug resistance in MDM2 over-expressing cells due to increased Akt phosphorylation. Further investigation is required at this point. We also demonstrate the ability of MDM2 to increase processing and nuclear translocation of NF κ B2. By silencing NF κ B2, we show that the Paclitaxel resistance offered by MDM2 is diminished and this therefore being the route through which MDM2 increases resistance to Paclitaxel. Finally, we show the role played by NF κ B2 in proliferation, thus providing a plausible mechanism of MDM2 mediated increased proliferation.

M. Discussion:

MDM2 over-expression occurs in nearly 7% of all cancer types(15). Biochemical and genetic studies have shown a relationship between MDM2 over-expression and cancer development. MDM2 is also used as a prognostic marker in the clinic and is an important target for anti cancer drug development(133). However no significant treatment strategy has been identified in cancers where MDM2 is over-expressed.(141) Most of the studies involving MDM2 attribute its oncogenic potential to its ability to bind with and degrade the tumor suppressor p53. However MDM2 has p53 independent activities and it has been shown to be oncogenic in permissible environments(54). This leads to the question of what mechanisms are employed by this oncogene towards oncogenesis.

A lot of recent work has shown the involvement of MDM2 with the mitogenic signaling pathways. MDM2 has been shown to be phosphorylated by Akt at Serine 166,186 and 188(67,85,86). This event results in the nuclear translocation of MDM2 and further binding and degradation of p53; phosphorylation of MDM2 by Akt is thought to be necessary for degradation of p53 and a mechanism of oncogenesis. MDM2 is known to shuttle between the nucleus and cytoplasm and has also been shown to have activities outside the nucleus. Examples of this include degradation of cell surface receptors like IGFR(68), Androgen receptor(81) by MDM2, binding to and degrading signaling molecules like GRK-2(70) and β Arrestin (69). The current thinking behind these activities of MDM2 is that after nuclear entry and its interaction with p53, MDM2 gets exported out of the nucleus and the excess MDM2 is available for the above interactions(69,70). However, there is no study to indicate the sequence of events when the cell lacks p53.

Although much has been done to show the role of activated Akt pathway in the nuclear entry of MDM2, there is no explanation for situations in which there is over-expression of MDM2 in the cell. We have observed that a transient over-expression of MDM2 shows most of the expression within hours of transfection, reaching a peak at about 8 hours and then the levels going down. It has been suggested that MDM2 protein levels are regulated by self-ubiquitination, which happens after nuclear entry and subsequent export. An intuitive suggestion would be that enhanced levels of Akt activation would be required for phosphorylation of the increased pool of MDM2 to facilitate its nuclear entry, which precedes its export. This scenario is depicted in Figure 29,

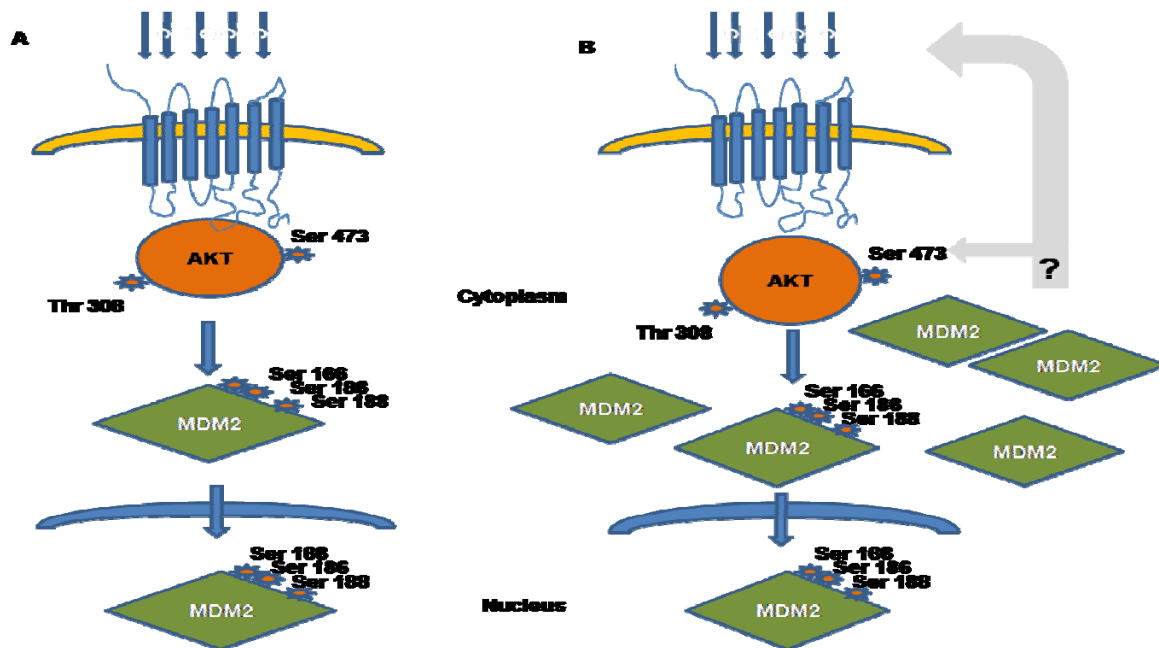


Figure29. MDM2 and Akt phosphorylation. (A) The interaction of MDM2 and phosphorylated Akt results in the phosphorylation of MDM2 and subsequent nuclear entry. (B) When MDM2 is over-expressed, the pool of phosphorylated Akt will be used up to drive the nuclear entry of MDM2, and the unphosphorylated MDM2 which remain in the cytosol may demand for increased Akt phosphorylation.

Our observation that MDM2 mediates Akt phosphorylation represents a novel signaling event. Both cancer cell lines and normal diploid fibroblasts show enhanced Akt phosphorylation when MDM2 is over-expressed and silencing of endogenously over-expressing MDM2 in cancer cell lines reduces the Akt activation. Also importantly, this is an event that is independent of two of the most important tumor suppressors- p53 and pTEN, as H1299 cell lines lacks p53 and has suppressed PTEN expression(210,211).

We observed that MDM2 over-expressing cells could offer enhanced chemo resistance to drugs used in the clinical setting against various cancer subtypes. Several mechanisms have been put forth to explain the possible role for MDM2 in decreasing response to chemotherapy. Besides the most well known ability to degrade p53 and therefore reduction in apoptosis and cell cycle arrest, MDM2 has been shown to increase the expression of the Multidrug Resistant gene (mdr1) atleast in glioblastoma cell lines(212). Other groups have shown the ability of MDM2 to increase p65 expression and therefore cause Doxorubicin resistance(80). Here we show that by inhibiting the Akt phosphorylation we can sensitize MDM2 over-expressing cells to treatment by Etoposide. Since there is no treatment strategy available at present when MDM2 over-expression is used as marker, targeting the Akt pathway in these tumors could be pursued as a viable treatment technique. Also whereas most of the current work focusing on small molecule inhibitors, peptide blockers and RNA interference against MDM2 has been shown to induce the apoptotic cell death in normal cells, targeting the Akt pathway has been shown to be more specific(213). If more data is generated in this scheme, we will be able to have a clearer picture on this treatment strategy and work on its short comings.

We also show that inhibition of Akt phosphorylation serves to counter the enhanced proliferation rate in cells over-expressing MDM2. Further we show that MDM2 expressing cells have increased migration ability. Since the cell processes of increased proliferation, migration and chemo resistance are properties that define the nature of cancer cells, it seems to suggest that MDM2 mediated oncogenesis could occur through the activation of the Akt pathway.

Towards finding a mechanism of Akt activation by MDM2, we studied the importance and requirement of the PI3K pathway and found that inhibition by wortmannin can inhibit the Akt phosphorylation by MDM2. Also we see enhanced IGF-1 signaling occurring when cells over express MDM2 and when endogenous over-expressed MDM2 is silenced, it reduces the IGF-1 signaling pathway. Since the IGF-1 has been shown to activate Akt through the PI3K pathway(169-172), this suggests that MDM2 mediated Akt phosphorylation happens through the PI3K pathway. Also it suggests that MDM2 may be augmenting some factor in the signaling pathway, since silencing MDM2 reduces the signaling mechanism. However there is no change in the levels of Threonine 308 phosphorylation by MDM2 which may be a counter argument for the role of the PI3k pathway(100,101,163,164,173). This may be explained in terms of the deregulation of the PDK1 in H1299, which serves to phosphorylate Akt at Threonine 308 or some other member in the pathway. Further investigation is required for a more substantial answer. Another supporting evidence for the role of the PI3K pathway comes from our observation that Rictor, a kinase which has been shown to phosphorylate Akt at Serine 473 residue specifically(99) does not play any role in MDM2 mediated Akt activation. Also there is no

change in transcript levels of PHLPP, a phosphatase specific for serine 473 residue(106,107) and CTMP, a protein that binds to Akt and dephosphorylates it(109). It should be taken into consideration that Akt phosphorylation at serine 473 can be performed by other cellular kinases like ILK(100-102), DNA-PK(104) when there is DNA damage as well as other, still debatable kinases. A more definite answer would require substantial work to be done for the proper mechanism to be charted out. An additional insight on the mechanism can be got from the ability of MDM2 to increase transcript levels of CXCL8 or IL8. This has been shown to be able to activate the Akt pathway. Also it falls in place with our migration data(177). This needs further investigation and could prove to be the mechanism.

MDM2 has been shown to interact with a wide variety of proteins in the cell and modulate their expression and thereby cellular processes. These interactions have been mapped to the various domains of MDM2 and have served the purpose of providing valuable clues in identifying the properties of MDM2 and their mechanism(54,166). Our domain analysis resulted in the identification of a domain stretching from amino acids 189-220, which has been shown to be the region where MDM2 and phosphorylated Akt interact(86), to be responsible for our observation. The domain analysis also allowed us to negate the possibility of our observation to be caused by one of our previous observations in the laboratory- MDM2's interaction with Elongation Factor EF1 α . This interaction required two domains lying between amino acids 1-58 and another between amino acid 221-325(82) and MDM2 mediated Akt phosphorylation does not seem to require the first 154 amino acids at least. Also we showed that deletion mutants able to induce phosphorylation

of Akt, increase resistance to Etoposide and inhibition of the Akt phosphorylation in these mutant expressing cells, sensitizes them to drug treatment similar to what we see with full length MDM2.

In trying to figure out the consequences of MDM2 mediated Akt phosphorylation, we looked at one of the most frequently studied downstream target of Akt phosphorylation, GSK3(126-128,180,181) and found that MDM2 expressing cells express higher levels of phosphorylated GSK3 α and β . These levels can be reduced by using an inhibitor targeting Akt, thus proving the importance of the elevation of Akt activation towards increasing phosphorylated GSK3 levels. Also silencing endogenous levels of MDM2 in over-expressing cell lines reduces the GSK3 phosphorylation. We also did a qPCR array to identify some of the targets, shown to be involved in the Akt pathway that get modulated by MDM2 induction in an ponasterone inducible clone. Our result showed changes in expression of genes which have been identified to be important in processes of proliferation, migration, apoptosis resistance, chemotherapeutic resistance among others. The most significant changes were shown by genes which controlled apoptosis with Bcl2(113), an anti apoptotic factor showing nearly 96 fold increase. Therefore we validated this result and saw that MDM2 over-expressing cells indeed expressed higher transcript and protein levels of Bcl2. Also, silencing of MDM2 reduced Bcl2 levels. Consistent with the ability of activated Akt to induce processing of NF κ B2(205), we observed that MDM2 over-expressing cells had higher levels of processed NF κ B2. Also we show that MDM2 over-expressing cells have higher nuclear localization of NF κ B2. This is important because NF κ B2 is a well known transcription factor and has been shown to activate genes

responsible for proliferation, migration, and drug resistance and anti apoptosis including Bcl-2(206-209,214). Towards finding the role of NFκB2 in MDM2 mediated chemo resistance; we did clonogenic assays after silencing NFκB2 and present evidence that the MDM2 mediated Paclitaxel resistance goes through the NFκB2 route. However lowering of NFκB2 does not compromise MDM2 mediated resistance to Etoposide. Further work will be required to elucidate the exact mechanisms of drug resistance to Etoposide and Paclitaxel and it will be interesting to figure out the distinct pathways by which MDM2 provides resistance to various chemotherapeutic agents.

N. Experimental Methods.

Plasmids and siRNA: The MDM2 cDNA was a generous gift from Bert Vogelstein (Johns Hopkins University Medical Institutions, Baltimore, MD) (215). Construction of plasmids expressing full-length MDM2 and its deletion mutants has previously been described in detail ((45,47)). Plasmids (pLKO.1) expressing short hairpin (sh) RNA against MDM2 and NF- κ B2 from U6 promoters and harboring puromycin resistance gene were purchased from Open Biosystem. The control plasmid expresses shRNA against non-endogenous luciferase gene. Plasmid (pLKO.1) expressing short hairpin (sh) RNA against Rictor from U6 promoter, Rictor-1 was purchased from Addgene. The Short interfering (si) RNA, siGENOME SMARTpool reagent M-003279-02-0005, directed against human MDM2 (MDM2 siRNA) was purchased from Dharmacon, si RNA against NF κ B2 (duplex of 5'-rgrCrCrCUrgrArgUrgrCrCUrgrgrAUrCU TT-3' and 5'rArgrAUrCrCrArgrgrCrArgrgrgr TT-3') and control scramble RNA (duplex of 5'-rCrAUrgUrCrAUrGUrgUrCrArCrAUrCUrC TT -3' and 5'rgrArgrAUrgUrgrArCrArCrAUrgrArCrAUrG TT-3') was designed using a program suggested by Qiagen Inc, and was purchased from Proligo.

The pIND β globin intron MDM2 used for generating the inducible clones was generated by restriction digestion of the pIND β globin vector (Invitrogen) using BamH1 and ligating it with the MDM2 cDNA. This clone was designated the pIND β globinMDM2.

Cells, transfections and generation of stable transfectants: H1299, MCF10A and WI38 cells were purchased from American Type Culture Collection and were maintained in media as suggested by the suppliers. SJSA-1 cell line was a kind gift from A.T. Look

(216). HEK 293T cells was a kind gift from Dr Qui Y (University of Maryland) For nucleofection, cells were seeded 48 to 72 hours before transfection. Cells were then trypsinized, and a suspension of 3×10^6 cells was mixed with plasmids or siRNA and pulsed (nucleofected) using a Nucleofector and kit reagents (Amaxa) following supplier's protocol and plated in 10cm culture dishes in normal growth media. To generate MDM2 stable transfectants, H1299 cells were lipofected with MDM2 expression plasmids (full-length or deletion mutants) or vector plasmid harboring neomycin resistance gene using lipofectamine 2000 reagent(Invitrogen) as per manufacturer's protocol. The cells were pooled and selected using 100 μ g/ml of G418 (GibcoBRL). To generate stable transfectants expressing MDM2 shRNA, SJSA-1 cells were transfected with pLKO.1 plasmids expressing MDM2 shRNA or luciferase shRNA by nucleofection. Nucleofected cells were selected with pooled and selected using 2 μ g/ml puromycin (Invivogen). Several MDM2 shRNA expression plasmids were tested to select expression vectors efficiently silencing MDM2 expression.

Antibodies: Antibody against MDM2 (IF-2) from Calbiochem or N-20 (SC813) from Santa Cruz was used in 1:100 dilutions. 2A10 antibody was a gift from Arnold Levine and was used at 1:200 dilutions. Antibody against, Akt phosphorylated at Ser473 (#9271) and Thr 308(#9275), Rictor (#2114), GSK3 α/β phosphorylated at ser 21/9 (#9331) were purchased fromCell Signaling and used as per manuufacturer's instructions., β -actin antibody (AC-15) was purchase from Sigma and used at 1:5000 dilution, CDK4 antibody Ab-2 was purchased from Calbiochem and used at 1:1000 dilution. Akt (sc-8312), GSK3 α/β (sc-56913), Sp1(sc-59 1)and GAPDH (sc32233) was purchased from Santacruz

Biotechnology and used at 1:1000 dilution, NF- κ B2 (#05-361) was purchased from Upstate Biotechnology and used at 1:5000 dilution, pERK(V-8031) was purchased from Promega and Bcl2 (610538) from BD biosciences and used as per manufacturer's protocol.

Western Blot Analysis: Cells were washed with ice-cold phosphate buffered saline (PBS) and harvested in a cell lysis buffer (Promega) containing 50 mM NaF, 0.1M phenylmethylsulfonyl fluoride, Na pyrophosphate, Na orthovanadate and protease inhibitors as described previously (9, 18). The lysate was boiled for 5 minutes in 4X lamellae loading buffer dye (50 mM Tris-HCl pH 6.7, 2% SDS, 2% β -mercaptoethanol, and bromophenol blue) and analyzed by SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis using respective antibodies ((217)).

For westerns involving detection of phosphorylated Akt, the plates were washed with ice cold PBS once and harvested in 1 ml ice cold PBS by scrapping into tubes. They were spun at 990g, 10 mins, 4 °C and the pellets treated with lysis buffer consisting of 187.5mM Tris-HCl(pH6.8 at 25 °C), 6%w/v SDS, 30% Glycerol, 150mM DTT and 0.035 w/v bromophenol blue and sonicated for 15 secs,(5 secs on, 2secs off pulses) at output of 3 in a Misonix 3000 sonicator using a microtip. The tubes were spun down, boiled and analyzed by SDS-polyacrylamide gel electrophoresis using a 10% acrylamide gel, followed by Western blot analysis using respective antibodies.

Inhibitors and chemotherapeutic drugs: Wortmannin (Upstate Biotechnology) treatments were carried out at a concentration of 100 nM for 4 hours ((121,218)). Akt inhibitor SH-6 (AKT III) (Calbiochem) was used at a concentration of 1.76 μ M for 4

hours. After pretreatment, cells were either harvested for Western blot analysis or plated to determine drug sensitivity by colony formation assay. IGF-1 (#I3769) was purchased from Sigma and used at 50ng/ml final concentration. Etoposide, Carboplatin and Paclitaxel were purchased from Sigma and used at appropriate concentrations.

Determination of drug sensitivity by colony formation assay: Colony formation assay was performed as reported earlier (219). Cells were seeded at equal density in 10-cm plates in triplicate and treated with chemotherapeutic drugs at specified concentration or the vehicle for 48 hours. Cells were then grown in normal growth media to allow colony formation by viable cells. After generation of colonies cells were fixed with methanol, stained with methylene blue and colonies were counted.

Growth Assay: Cells were seeded at a density of 1×10^4 cells per 60-mm plate. Fifteen plates of each line were plated initially. Whenever needed three plates of each was trypsinized and cells were suspended in 1 ml of serum containing medium. Cells were counted using a Beckman Coulter Counter and the total number of cells calculated and plotted. Media in the remaining plates were changed every day. Standard deviations were calculated from the three individual experiments run simultaneously.

Lentivirus generation and infections: Lentiviral production was done by co-transfection of pDelta8.7 backbone, pVSVG and the pLKO.1 vectors expressing the shRNA into 293T cells by calcium phosphate transfection(220). The media was harvested after 48 h and aliquoted. It was subjected to flash freezing and further storage at -80°C .

Cells were seeded at a density of 2×10^5 in 10cm plates, 24 hours prior to the infection. For infection, the media was removed and the cells were washed once with Hanks Balanced

Salt Solution. The frozen virus was thawed quickly and added to the plates. The plates were rocked every 15 minutes by hand and incubated at 37 °C. After 2 hours fresh media containing 10% serum was added and kept in the incubator at 37 °C, 5% CO₂.

Migration assay: For the scratch assay, cells were trypsinized using 0.1% trypsin-2.21mM EDTA (Mediatech, Herndon, VA) and plated in triplicate in a 12-well cell culture plate (Greiner Bio-One, Monroe, NC) and incubated at 37° C until cells were completely confluent. At this time, a sterilized pipette tip was placed within each well and scratched across the surface of the plate removing the complete layer of cells within the scratch area. Following cell removal, each well was washed once with PBS and then replaced with medium. Immediately following, the width of the scratch was measured at a specific point under a 5x objective using a light microscope and AxioVision software. Cells were then returned to a 37° C incubator. Cells were incubated for a total of 20 h, at which time the scratch width was measured again using the same procedure and at the same position as in time 0.

Inducible clone generation: Ponasterone inducible clones were generated as per manufacturer's protocol (Invitrogen). Briefly, H1299 cells were plated one day previous to the transfection in a 6well plate. The next day, co transfection was conducted on these cells with pvgRXR (Invitrogen) and the pINDβglobinMDM2 using Lipofectamine2000(Invitrogen) using manufacturer's protocol. After 24 hours the cells were trypsinized and re-plated in a 10cm plate and selected using 100μg/ml G418(Gibco) and 25μg/ml Zeocin(Invitrogen). Clones were then picked and allowed to undergo further selection and grow and analyzed by induction with 5μg/ml Ponasterone A(Invitrogen).

RNA extraction Generation of cDNA and quantitative PCR (QPCR): Total RNA was isolated from exponentially growing cultured cell lines using TRIzol reagent (Life Technologies, Invitrogen) following a protocol supplied by the manufacturer. Quality of RNA was checked by 2% agarose Tris-borate-EDTA gel electrophoresis. cDNA was synthesized using the Thermoscript reverse transcription-PCR system (Invitrogen). QPCR was performed using a LightCycler system (Roche). Primers were designed using OLIGO 5 software (Molecular Biology Insights) and were synthesized by Sigma Genosys(MDM2, GAPDH) or IDT(CTMP,PHLPP). Reactions were performed in triplicate utilizing SYBR green dye, which exhibits a higher fluorescence upon binding of double-stranded DNA. The methods have been described previously (219). The QPCR primers used were as follows:

- (a) MDM2, 5'- CCC AAG ACA AAG AAG AGA GTG TGG- 3' and 5'- CTG GGC AGG GCT TAT TCC TTT TCT- 3';
- (b) GAPDH, 5'-GTC AAC GGA TTT GGT CGT ATT-3' and 5'- GAT CTC GCT CCT GGA AGA TGG-3',
- (c) CTMP,5'-CTAAGACTGCTCTTTGACCAG 3' and 5'- CTCCATGAATGAATCCAGGT-3'
- (d) PHLPP, 5' CACCCCATCTGTCTGTCCA3' and 5'GCTGCTGTTTCTCCTTCTCC3'.
- (e) Bcl2, 5'- CAACATCGCCCTGTGGAT-3' and 5' GCCAAACTGAGCAGAGTCTTC3'

(f) CXCL8, 5'-TTTTGCCAAGGAGTGCTAAAG-3' and 5'-

AACCCTCTGCACCCAGTTTTC-3'

(g) Actin, 5'-CATGTACGTTGCTATCCAGGC-3' and 5'-

CTCCTTAATGTCACGCACGAT-3'.

PCR-Array: The Ponasterone inducible clone cells were induced for 24 hours and RNA extracted and cDNA generated as mentioned earlier PCR was performed in a Human Signal Transduction Pathway Finder RT² ProfilerTM PCR Array(SuperArray Bioscience Corporation) in a ABI Prism 7500 Sequence Detector (Applied Biosystems). For data analysis the $\Delta\Delta C_t$ method was used; for each gene fold-changes were calculated as difference in gene expression between induced and uninduced cells.

Preparation of cytoplasmic and nuclear extracts: Cytosolic and nuclear extracts were prepared following a method by Mendez and Stillman (221) Cultured cells were harvested by trypsinization, washed with phosphate buffered saline and were resuspended in a buffer A containing 10 mM HEPES (ph 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 1X protease inhibitor (Calbiochem), and 0.1 mM PMSF (phenyl methyl sulfonyl fluoride, Sigma). Cells were lysed by addition of Triton X-100 to a final concentration 0.1%, and incubating on ice for 5 minutes. Nuclei were collected by low speed centrifugation (4 min, 1,300g, 4°C). The supernatant were further clarified by high-speed centrifugation (12,000g, 4°C). The nuclear pellet was washed in buffer A and then lysed in a buffer B (3 mM EDTA, 0.2 mM EGTA, 1mM DTT and protease inhibitors as described above. Insoluble chromatin fraction was collected by centrifugation (4 min, 1,700g, 4°C), washed once with buffer B, suspended in Laemmli loading buffer and

sonicated for 30secs(5secs on and 1 sec off pulse) at output of 3.5 using a Misonix 3000 sonicator using a microtip

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R. Vitae

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